ORGANIC ANION-TRANSPORTING POLYPEPTIDE (OATP) FAMILY: CONTRIBUTION OF GENETIC VARIATION AND BOTANICAL INTERACTIONS TO VARIABILITY IN DRUG DISPOSITION AND RESPONSE

By

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Dedicated to the two loves of my life – my husband, Matthew A. Stanton and our son-on-the-way, Wesley M. Stanton

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ABC	ATP-binding cassette transporter family
AUC	Area under the plasma concentration time curve
BCRP	Breast cancer resistance protein
CL/F	Apparent oral clearance
C_{max}	Maximum concentration
EGCG	Epigallocatechin gallate
ENA-78	Epithelial cell-derived neutrophil-activating peptide 78 protein
HDL	High-density lipoprotein
hs-CRP	High-sensitivity C-reactive protein
IL-1RA	Interleukin 1 receptor, type 1
λ_z	Elimination rate constant
LDL	Low-density lipoprotein
MCP-1	Monocyte chemotactic protein-1
MRPs	Multidrug resistance-associated proteins
OAT	Organic anion transporters
OCT	Organic cation transporters
OATP	Organic anion-transporting polypeptide
P-gp	P-glycoprotein
SLC	Solute carrier transporter family
t_{max}	Time of maximum concentration
TPO	Thrombopoietin
V_{ss}	Volume of distribution at steady-state

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Drug transport plays an important role in the disposition of many drugs through effects on absorption, distribution, metabolism, and elimination. Ultimately, these effects on pharmacokinetics contribute to variability in drug response. For instance, members of the organic anion-transporting polypeptide (OATP) family, which facilitate the uptake (absorption and/or elimination) of a large variety of drug substrates, are now increasingly being recognized as a key determinant of drug disposition and can have a critical impact on drug response. Consequently, drugs are now screened during the development process to identify drug transporter substrates. As more drugs are being recognized as substrates for OATP transport, it is imperative that we fully understand the impact of these transporters on drug disposition and response. The goal of this work was to evaluate the effects of genetic variability and a botanical interaction on OATP-mediated transport. First, we evaluated the effects of OATP1B1 polymorphisms on drug response by conducting a retrospective pharmacogenetic analysis of atorvastatin-treated individuals. We hypothesized that OATP1B1 reduced-function carrier status would be associated with diminished lipoprotein reduction and enhanced inflammatory

marker reduction as compared to non-carriers. However, we did not observe any association between OATP1B1 reduced-function carrier status and atorvastatin response. Next, we conducted a clinical pharmacokinetic study to evaluate the inhibitory effect of green tea extract on OATP-mediated transport using the OATP probe fexofenadine. We hypothesized that fexofenadine exposure would be altered after a single dose of green tea extract due to decreased OATP-mediated uptake. Interestingly, we observed significantly lower fexofenadine plasma concentrations in the presence of green tea extract suggesting inhibition of intestinal OATP-mediated uptake. Future research to investigate the role of green tea extract in transporter-mediated interactions and the resulting clinical implications is warranted.

CHAPTER 1
OVERVIEW OF DRUG TRANSPORTERS AND EFFECTS ON DRUG DISPOSITION AND RESPONSE

Introduction

Drug transporters are membrane proteins expressed throughout the body that control the influx and efflux of many endogenous and exogenous substrates, including drugs. The primary role of these transporters is to transport nutrients and endogenous substrates and to protect the body from toxins. However, drugs that have structural characteristics similar to these substrates may also be transported. As a result, these transporters can have a significant impact on drug response as they can influence the absorption, distribution, metabolism, and elimination of drugs in the body. Through these influences on drug disposition, transporters can ultimately affect steady-state concentrations and contribute to variability in drug response. Recognition of the important role of drug transport in drug disposition and response is increasing. Of particular interest is the variability in transporter function and expression, which may contribute to interindividual variability in drug disposition and response. Transporter function and expression may be altered in the presence of disease, genetic variation, or drug interactions due to concomitant drugs, foods, and dietary supplements.

Drug transporters have been implicated in numerous drug-drug interactions, food-drug interactions, and herb-drug interactions (Han, 2011). For this reason, the Food and Drug Administration (FDA) stated in the most recently published drug interaction guidance document, *Drug Interaction Studies-Study Design, Data Analysis, and Implications for Dosing and Labeling*, that drug transporter analysis will be an integral part of the drug evaluation process and called for improved methodologies to investigate drug transporter interactions (U.S. Department of Health and Human

Services, 2012). Accordingly, the International Transporter Consortium (ITC), a partnership between representatives of the FDA, industry, and academia, have provided recommendations to identify new molecular entities that are substrates and/or inhibitors of drug transporters (Giacomini et al., 2010).

Most drug transporters can be classified into two families, either the Solute Carrier Superfamily (SLCs) or the ATP-Binding Cassette Superfamily (ABCs). The ABC transporters are primary active transporters that have an ATP binding domain and utilize ATP hydrolysis to transport substrates across membranes. Conversely, SLC transporters do not have ATP binding domains and transport by either facilitated diffusion, transport with the electrochemical potential gradient, or secondary active transport, using symport or antiport in the presence of active transport to transport against the concentration gradient.

Uptake Transporters of the Solute Carrier Superfamily

Organic Anion-Transporting Polypeptides

Organic anion-transporting polypeptides (OATPs) are one of the most widely investigated transporters due to their broad substrate specificity and important role in the disposition of many drugs. OATPs are classified into families and subfamilies based upon sequence homology, in which members of families and subfamilies share greater than 40% and 60% sequence homology, respectively. The nomenclature used for the transporter proteins and genes is based upon the family/subfamily classification system (Hagenbuch and Meier, 2004). For instance OATP1B1 is member 1 of subfamily B in family 1 and OATP1B3 is member 3 of the same family and subfamily. Likewise, the *SLCO* genes that encode for OATPs maintain the same nomenclature (e.g., *SLCO1B3* encodes the OATP1B3 protein).

The OATP transporters that have been found to be most clinically relevant are OATP1B1, OATP1B3, OATP2B1, and OATP1A2. The OATP1B1 and OATP1B3 transporters, which share 80% sequence homology, are both predominantly expressed on the sinusoidal membrane of hepatocytes (Konig et al., 2000a; Konig et al., 2000b). These transporters facilitate the hepatic uptake of drugs, which is a critical first step in elimination. OATP2B1 is expressed in hepatocytes as well as the intestines (Kullak-Ublick et al., 2001; Kobayashi et al., 2003) and OATP1A2 is expressed in the brain, liver, kidney, and intestines (Kullak-Ublick et al., 1995; Glaeser et al., 2007). OATP transporters that are expressed in the intestines facilitate drug absorption.

The OATP transporters have some overlap in terms of substrate specificity and share many endogenous as well as exogenous substrates, most notably statins. For many statins, OATP transport is particular important as the site of action and elimination (metabolism) occur in the liver (Kalliokoski and Niemi, 2009). OATPs also transport bile acids, bilirubin, conjugated steroids, certain antidiabetic medications, and fexofenadine (Table 1-1). A number of compounds are also known to inhibit OATPs, such as cyclosporine, rifampin, erythromycin, and fruit juices (Bailey et al., 2007; Niemi, 2007; Reitman et al., 2010). However, there are no known OATP inducers.

Due to the broad substrate specificity and number of potential inhibitors of OATP, numerous drug interactions have been reported involving OATP-mediated uptake in the intestines (absorption) and liver (elimination). For instance, the area under the concentration-time curve (AUC) of the antihypertensive drug aliskiren was reduced by approximately 60% when administered with grapefruit, orange, or apple juice due to intestinal OATP inhibition (Tapaninen et al., 2010a; Tapaninen et al., 2010b; Rebello et

al., 2011).Conversely, hepatic OATP inhibition may result in increased AUC since clearance is being inhibited due to reduced hepatic uptake. For instance, several fold increases in statin exposure (AUC) have been observed in the presence of the known OATP inhibitor cyclosporine (Simonson et al., 2004; Neuvonen et al., 2006). This magnitude of an effect on drug disposition can result in serious toxicities as systemic exposure is greatly increased.

Significant increases in statin exposure have also been reported in the presence of genetic variation (Niemi et al., 2004; Pasanen et al., 2006; Ho et al., 2007; Pasanen et al., 2007; Deng et al., 2008). The *SLCO1B1* 521T>C SNP is associated with reduced OATP1B1 function resulting in increased systemic exposure. In a clinical pharmacokinetic study, atorvastatin exposure (AUC) was 61% and 144% greater in heterozygous and homozygous carriers of the 521C allele, respectively (Pasanen et al., 2007). A genome-wide association study in the SEARCH trial evaluated approximately 300,000 markers in individuals with simvastatin-induced myopathy and the 521C allele was attributed to over 60% of the myopathy cases (Link et al., 2008). In addition to statin toxicity, *SLCO1B1* SNPs that are in linkage with 521T>C have also been associated with enhanced clearance of the anticancer drug methotrexate and increased risk of gastrointestinal (GI) toxicity in children being treated for leukemia (Treviño et al., 2009). However, in this case the 521C allele was associated with reduced clearance and thought to be protective against methotrexate GI toxicity. The 521T>C SNP has also been associated with significantly higher exposure of repaglinide (Niemi et al., 2005a; Kalliokoski et al., 2008b; Kalliokoski et al., 2008c). In a study of healthy volunteers who received a single oral dose of repaglinide, the AUC was 188% higher in

521CC carriers as compared to TT carriers (Niemi et al., 2005a). In another healthy volunteer study, the 521T>C was again found to be associated with increased AUC and there was a trend toward enhanced glucose-lowering correlated to AUC (Kalliokoski et al., 2008b). Conversely, the *SLCO1B1*1b* haplotype, which is associated with enhanced uptake, has been associated with reduced exposure of repaglinide, consistent with increased hepatic uptake (Kalliokoski et al., 2008a).

Polymorphisms have also been identified in the *SLCO1B3*, *SLCO2B1*, and *SLCO1A2* genes (Zaïr et al., 2008; Franke et al., 2009; Sissung et al., 2010). In a pharmacokinetic study in renal transplant patients receiving the immunosuppressant mycophenolate, the *SLCO1B3* 334T>G SNP was evaluated and the GG genotype was found to be associated with lower oral clearance of the metabolite mycophenolic acid (Miura et al., 2007a). The investigators concluded that the *SLCO1B3* 334T>G SNP explains in part the high interindividual variability in mycophenolate pharmacokinetics. At this time, the function of *SLCO1A2* SNPs and their effects on pharmacokinetics and drug response are not well understood (Franke et al., 2009). However, three *SLCO1A2* SNPs within the promoter region show trends of altered clearance of the anticancer drug imatinib (Yamakawa et al., 2011).

Organic Cation Transporters

Organic cation transporters (OCTs), first identified in the kidney, are members of the *SLC22A* family. OCTs are primarily expressed in the kidney and liver and facilitate the influx of low molecular weight cations across the basolateral membranes of renal proximal tubules and hepatocytes (Koepsell et al., 2007). OCT2 is considered to be kidney specific and OCT1 is liver specific, although OCT1 is also expressed in the intestines. At the basolateral membrane of the renal proximal tubule, OCT2 fluxes drug

from the blood into the tubule, which is the first step in active secretion prior to elimination from the kidney. Similarly, OCT1 is involved with the first step of hepatic elimination by fluxing drug from the blood into the hepatocytes. The final member, OCT3, is broadly expressed, but is not recognized as highly important in drug disposition and response as OCT2 and OCT1.

The OCTs share some substrate specificity and transport creatinine, metformin, procainamide, oxaliplatin, as well as other drugs. OCT3 is involved with the transport of monoamine neurotransmitters. Rifampin, a pregnane X receptor agonist, has been identified as an OCT1 inducer (Cho et al., 2011). Drugs that have been identified as inhibitors include cimetidine, quinidine, and pilsicainide (Koepsell et al., 2007; Ciarimboli, 2008).

A number of drug interactions resulting in reduced renal clearance due to OCT inhibition have been reported. A case report of an interaction between the OCT inhibitors certirizine and pilsicainide has been described in which increased plasma concentrations of pilsicainide resulted in severe arrhythmia in an individual with existing renal insufficiency (Tsuruoka et al., 2006). The authors then evaluated this interaction in a clinical pharmacokinetic study in healthy volunteers and observed reduced renal clearance for both certirizine (38% decrease) and pilsicainide (41% decrease) when the drugs were coadministered (Tsuruoka et al., 2006). Many drug interactions with the OCT inhibitor cimetidine have also been reported. For example, cimetidine inhibits renal clearance of the OCT substrate metformin. In a clinical pharmacokinetic study in healthy volunteers, the AUC of metformin was increased by 50% and renal clearance decreased by 27% after cimetidine administration (Somogyi et al., 1987).

OCT2 has been found to be particularly important to the elimination of metformin as metformin is renally excreted primarily by active secretion. However, there is evidence that OCT1 is also significant to metformin elimination. Polymorphisms within *SLC22A1* and *SLC22A2* affect metformin disposition and response (Shu et al., 2007; Shu et al., 2008; Song et al., 2008; Becker et al., 2009; Chen et al., 2009). In a healthy volunteer study, individuals with genotypes associated with reduced-function OCT1 had higher metformin AUC and C_{max} values as compared to individuals with the reference genotype (Shu et al., 2008). The authors, Shu et al., also investigated the effect of OCT1 polymorphisms on plasma glucose levels in healthy volunteers during oral glucose tolerance testing (OGTT) when metformin is administered. They found that both reference carriers and variant carriers of OCT1 polymorphisms had similar glucose levels after OGTT in the absence of metformin. However, in the presence of metformin, the variant carriers had 17% higher glucose AUCs as compared to the reference carriers. In addition, metformin produced significant reductions in glucose AUC as compared to the control phase in reference carriers, but not in variant carriers (Shu et al., 2007). Effects of OCT1 polymorphisms have also been evaluated in diabetic patients. In a study evaluating HbA1C in diabetic patients being treated with metformin, the OCT1 ANP rs622342 A>C was associated with glucose lowering in that for each C allele, the reduction in HbA1C was 28% less (Becker et al., 2009).

Likewise, in another healthy volunteer study in an Asian population, OCT2 variant carriers (808G>T, 596C>T, and 602C>T) had significantly higher metformin AUC and C_{max} values and lower renal clearance as compared to the reference carriers, which is consistent with reduced renal uptake (Song et al., 2008). Conversely, in a similar study

in a European and African ancestry population, the variant carriers of the 808G<T SNP had significantly higher metformin renal clearance, indicating racial differences in OCT2 function (Chen et al., 2009).

Organic Anion Transporters

Organic anion transporters (OATs), like OCTs, belong to the *SLC22A* family and are predominantly expressed on the basolateral membrane of the renal proximal tubule, but OATs primarily transport small organic anions (Koepsell and Endou, 2004). OATs are also expressed in the blood-brain barrier, placenta, and other tissues. OAT1 and OAT3 are the best characterized of the OATs, are recognized as important in drug disposition, and have the highest expression of the transporters in the kidney (Hilgendorf et al., 2007).

OAT1 and OAT3 share broad and moderately overlapping substrate specificity. OAT drug substrates include steroid hormones, diuretics, angiotensin converting enzyme inhibitors, angiotensin receptor blockers, antibiotics, and antivirals (Rizwan and Burckhardt, 2007). Probenecid has been identified as an inhibitor of both OAT1 and OAT3 and has been implicated in OAT-mediated drug interactions (Laskin et al., 1982; Jaehde et al., 1995; Cundy, 1999). An interaction was reported with probenecid and acyclovir in a pharmacokinetic study in which acyclovir AUC increased by 40% and renal clearance decreased by 32% in the presence of probenecid (Laskin et al., 1982). Probenecid was also shown to reduce the renal clearance of cidofovir, but in addition, probenecid reduces the incidence of cidofovir-induced nephrotoxicity (Cundy, 1999). This beneficial interaction is thought to occur by blocking active tubular secretion of cidofovir and probenecid is now used clinically in conjunction with cidofovir for this purpose. On the other hand, reports of a life-threatening interaction thought to be

mediated by inhibition of OAT3 have been described, in which methotrexate toxicity occurred with coadministration of ketoprofen (Thyss et al., 1986; Takeda et al., 2002).

While OATs are evidently important in the disposition of many drugs that are dependent on renal clearance, there have been no reports of the effects of OAT genetic variation on drug disposition. However, a genetic variant has been associated with blood pressure response to hydrochlorothiazide. The intergenic polymorphism between *SLC22A6* and *SLC22A8*, which encode for OAT1 and OAT3, was evaluated in hypertensive patients receiving hydrochlorothiazide and greater blood pressure reduction was observed in the GG carriers versus the C allele carriers (Han et al., 2011). The authors concluded that this polymorphism may affect the expression or function of OAT1 and OAT3 and that OATs may have a role in blood pressure regulation.

Efflux Transporters of the ATP-Binding Cassette Superfamily

P-Glycoprotein

P-glycoprotein (P-gp), also known as multidrug resistance protein, is the most studied member of the ABC superfamily. P-gp was first recognized as an important determinant of drug disposition as its overexpression is associated to multidrug resistance in cancers (Sharom, 2008). Transport mediated by P-gp can have a major impact on the disposition and response of its substrate drugs. P-gp is widely expressed throughout the body at tissue barriers and through efflux prevents the absorption and accumulation of toxins and drugs. Specifically, P-gp is expressed on endothelial cells of the blood-brain barrier (BBB) where it can reduce the accumulation of drugs in the brain, the apical surface of enterocytes where it can reduce intestinal absorption, as well as placenta where it may act to protect the fetus (Choudhuri and Klaassen, 2006). In

addition to reducing absorption across biological barriers, P-gp is also expressed on the lumen of renal proximal tubules and at the bile canaliculus in hepatocytes where it can facilitate elimination by effluxing drugs into the urine and bile, respectively (Zhou, 2008).

P-gp has broad substrate specificity, including anticancer drugs, antivirals, immunosupressants, antiepileptics, steroids, analgesics and antihypertensives (Zhou, 2008). The efficacy of these substrate drugs can be severely reduced as P-gp can limit the absorption systemically and access to drug target sites. Verapamil, cyclosporine, ritonavir and quinidine are known p-gp inhibitor (Zhou, 2008). However drugs that can inhibit or modulate P-gp are being developed for the sole purpose of increasing the bioavailability and efficacy of P-gp substrate drugs. P-gp can also be induced by the pregnane X receptor (PXR) agonist rifampin (Urquhart et al., 2007).

Drug interactions due to P-gp inhibition and induction resulting in changes in pharmacokinetics and drug response have been reported. For instance, the P-gp inhibitor ritonavir has been shown to increase digoxin AUC by 86% and reduce renal clearance by 35% after coadministration of oral ritonavir and intravenous digoxin due to inhibition of P-gp resulting in reduced renal tubular elimination (Ding et al., 2004). An interaction between the P-gp inhibitor quinidine and the P-gp substrate loperamide in which respiratory depression occurred has also been reported (Sadeque et al., 2000). Loperamide is an opiate, but does not usually produce CNS effects as P-gp prevents penetration into the CNS. In this case, quinidine inhibited P-gp at the brain endothelium and allowed access of loperamide into the CNS as this interaction was not explained by changes in systemic loperamide concentrations.

Genetic variation in the *ABCB1* gene, which encodes P-gp, has also been associated with drug disposition and response (Ieiri, 2012). The most studied SNP is 3435C>T, which is commonly evaluated with the 2677G>T and 1236C>T SNPs as a haplotype. As P-gp has been implicated in resistance to antiepileptic drugs, the 3435C>T variation was evaluated in epileptic patients receiving phenobarbital (Löscher et al., 2009). The investigators observed equivalent phenobarbital serum concentrations among the CC, CT, and TT carriers, however, the variant homozygotes (TT) had higher phenobarbital CSF concentrations versus the C carriers. Additionally, CC carriers were found to have greater seizure frequency than the variant carriers. In this case, the variant was shown to be associated with greater drug penetration into the CNS as evidenced by increased CSF concentrations and greater efficacy. However, the observed associations with *ABCB1* genetic variation have been inconsistent overall in various disease states and populations (Chinn and Kroetz, 2007).

Breast Cancer Resistance Protein

Breast Cancer Resistance Protein (BCRP) is also associated with multidrug resistance and was first identified in cancer cell lines (Doyle et al., 1998; Miyake et al., 1999). BCRP is expressed in the intestines, kidney, liver, placenta, brain endothelium, and mammary tissue (Choudhuri and Klaassen, 2006). Like P-gp, BCRP also limits absorption across tissue barriers and facilitates elimination and thus is important to drug disposition and response. There is considerable overlap in substrates between BCRP and P-gp. BCRP substrates include anticancer drugs, antivirals, antibiotics, and statins (Choudhuri and Klaassen, 2006; Degorter et al., 2012). Estrone and 17β-estradiol are

known inhibitors (Giacomini et al., 2010). There is also interest in the development of BCRP inhibitors, such as the investigational P-gp/BCRP modulator elacridar.

In a phase 1 study in cancer patients, the effect of elacridar on the disposition of topotecan, a BCRP substrate, was studied (Kruijtzer et al., 2002). When topotecan was administered with elacridar, topotecan AUC and bioavailability increased by 140% and 97%, respectively. These results are promising as efflux transporters can significantly influence the efficacy of their substrate drugs, especially anticancer drugs.

Genetic variation of the gene that encodes BCRP, *ABCG2*, has also been shown to have effects on pharmacokinetics and efficacy (Ieiri, 2012). The effect of the most commonly studied *ABCG2* SNP, 421C>A, on topotecan pharmacokinetics was evaluated, and the investigators found that cancer patients carrying the variant allele had higher topotecan concentrations and bioavailability as compared to non-carriers when topotecan was administered orally (Sparreboom et al., 2005). However, this effect was not observed when IV topotecan was administered indicating reduced BCRP efflux in the intestine (improved bioavailability) of variant allele carriers. The effect of the 421C>A SNP on atorvastatin and rosuvastatin has also been studied .(Keskitalo et al., 2009). This study was conducted in healthy volunteers who each received a single oral dose of atorvastatin and rosuvastatin on two separate occasions. The investigators observed significantly higher AUC values for both atorvastatin and rosuvastatin in the homozygote variants (AA). The magnitude of effect was greatest with rosuvastatin in which AA carriers had AUC values 100% and 144% higher than CA and CC carriers, respectively. Further, the 421A variant has been associated with greater reduction in

LDL concentrations (improved efficacy) in hypercholesterolemic patients who received rosuvastatin (Tomlinson et al., 2010).

Multidrug Resistance-Associated Proteins

Multidrug Resistance-Associated Proteins (MRPs) were first identified in cancer cell lines and like P-gp and BCRP have been implicated in drug resistance to a wide variety of anticancer and antiviral drugs (Cole et al., 1992; Sharom, 2008).MRPs belong to the ABCC subfamily and include 9 members, however MRP2 has been the most studied in drug disposition. MRP3 and MRP4 are also considered to be important drug transporters. MRPs are primarily expressed in the intestine, liver, kidney, and brain (Choudhuri and Klaassen, 2006). MRP2 is localized on the apical membrane of these sites, MRP3 on the basolateral membrane of hepatocytes and renal proximal tubules, and MRP4 on the basolateral membrane of hepatocytes and the apical membrane of renal proximal tubules and the brain endothelium (Giacomini et al., 2010). MRP substrates include anticancer drugs, antivirals, glutathione and glucuronate conjugates, and bile acids (Choudhuri and Klaassen, 2006). Cyclosporine efavirenz, emtricitabine, and NSAIDs are known to inhibit MRPs while rifampin is known to induce (El-Sheikh et al., 2007; Urquhart et al., 2007; Giacomini et al., 2010).

MRP2 has been recognized as playing a role in drug-drug interaction and clinically relevant polymorphisms have been discovered. For instance, mycophenolate, a substrate of MRP2, has high interindividual variability in pharmacokinetics and some of this variability in part is due to variability in enterohepatic circulation of the metabolite mycophenolic acid. In a clinical study in patients receiving mycophenolate, it was observed that those who were also taking NSAIDS did not have evidence of enterohepatic circulation of mycophenolic acid as compared to those who were not

receiving NSAIDs (Fukuda et al., 2011). As NSAIDs have been shown to inhibit MRP2 efflux (El-Sheikh et al., 2007), the authors suggested that MRP2 inhibition by NSAIDs was the mechanism of this pharmacokinetic interaction. The effect of the MRP2 -24C>T SNP on mycophenolate pharmacokinetics was also evaluated (Lloberas et al., 2011). The investigators found that variant carriers had reduced mycophenolic acid exposure at steady-state as compared to the CC carriers. This effect was only observed in patients who were receiving tacrolimus and sirolimus as part of their immunosuppressant regimen, not in those receiving cyclosporine. However, cyclosporine is a MRP2 inhibitor (Hesselink et al., 2005) and the authors concluded that cyclosporine masks the effect of the genetic variation.

Transporter-Drug Metabolizing Enzyme Interplay

Due to the significant overlap in substrates between transporters and metabolic enzymes, it is important to also consider interplay as transporters can affect metabolism (Benet, 2009). P-gp efflux can affect intestinal absorption and therefore intestinal metabolism while OATP-mediated uptake in the liver can affect hepatic metabolism. The effects of rifampin (P-gp/CYP3A inducer and OATP inhibitor) on glyburide (substrate for P-gp, CYP3A, and OATP) pharmacokinetics were evaluated in a clinical study in which intravenous rifampin was administered with glyburide as a single dose before and after six days of oral rifampin (Zheng et al., 2009). Glyburide pharmacokinetics were analyzed before rifampin (control phase) and after the first IV rifampin dose, the six days of oral rifampin (induction phase), and the repeated IV rifampin dose after the induction phase. As compared to the control phase, the AUC of glyburide and its metabolite were significantly increased after the single dose of IV rifampin due to hepatic OATP inhibition (decreased clearance). Rifampin was then

administered orally for six days, adequate time to induce P-gp and CYP3A, and the AUC of glyburide and its metabolite were greatly reduced compared to the control phase due to induction of P-gp and CYP3A (reduced bioavailability). Interestingly, when the IV rifampin dose was repeated, hepatic inhibition masked the effects of P-gp/CYP3A induction and the AUC of glyburide and its metabolite were similar to the control phase.

Summary

In summary, the studies reviewed illustrate the importance of the role of drug transporters in disposition and response. Drug transporters have been implicated in drug interactions resulting in either sub- or supra-therapeutic concentrations, and correspondingly reduced efficacy or increased toxicity. Genetic variation can also be important as it can affect the expression and/or function of drug transporters. As more drugs are being recognized as substrates for transport and the importance of transport is increasingly realized, it is critical that we fully understand the impact of these transporters. This understanding will ultimately help guide us in predicting clinical efficacy, toxicity, and drug interactions. The overall goal of this work was to evaluate the effects of botanical interactions and genetic variability on OATP-mediated transport.

Study Objectives

- Determine the effect of organic anion transporting polypeptide 1B1 (OATP1B1) reduced-function carrier status on atorvastatin response. Our hypothesis was that reduced-function carrier status would be associated with diminished lipoprotein reduction and enhanced inflammatory marker reduction as compared to non-carriers.
- Characterize the effect of green tea extract (epigallocatechin gallate) on OATP-mediated drug transport by conducting a clinical pharmacokinetic study in healthy volunteers with the OATP probe fexofenadine. We hypothesized that fexofenadine exposure would be changed after a single dose of green tea extract due to decreased OATP-mediated uptake.

Table 1-1. SLC and ABC transporters important to drug disposition and response

	Selected substrates	Selected inhibitors	Expression
SLC transporters			
OATP1B1	Statins, repaglinide, valsartan, bilirubin, bile acids	Cyclosporine, rifampin, ritonavir	Liver
OATP1B3	Fexofenadine, telmisartan, statins, bile acids	Cyclosporine, rifampin, ritonavir	Liver
OATP1A2	Fexofenadine, methotrexate, levofloxacin, statins, bile salts	Naringin, rifampin, ritonavir	Intestines, liver, kidney, brain
OATP2B1	Fexofenadine, statins, glyburide	Cyclosporine, rifampin	Liver, intestines
OAT1	Zidovudine, lamivudine, tenofovir, ciprofloxacin	Probenecid	Kidneys, placenta
OAT3	NSAIDs, furosemide, bumetanide	Probenecid	Kidneys, brain
OCT1	Metformin, oxaliplatin	Quinine, quinidine	Liver, intestines
OCT2	Metformin, pindolol, ranitidine, varenicline	Cimetidine, certirizine	Kidneys
ABC transporters			
ABCB1	Digoxin, loperamide, doxorubicin, paclitaxel	Cyclosporine, quinidine	Intestines, kidneys, liver, brain
BCRP	Mitoxantrone, methotrexate, topotecan, imatinib, statins	Estrone, 17β-estradiol,	Intestines, liver, kidneys, brain, placenta, breast
MRP2	Glutathione and glucuronide conjugates, methotrexate	Cyclosporine, efavirenz, emtricitabine	Liver, kidneys, intestines
MRP3	Methotrexate, glucuronate conjugates	Efavirenz, emtricitabine	Liver, intestines
MRP4	Adefovir, tenofovir, furosemide,topotecan	Celecoxib, diclofenac	Kidneys, liver

Table 1-2. Selected transporter mediated drug interactions

Involved transporter	Perpetrator	Victim drug	Impact on victim drug	Reference
SLC transporters				
Hepatic OATP	Cyclosporine	Pravastatin	AUC ↑ 890%	(Neuvonen et al., 2006)
	Cyclosporine	Rosuvastatin	AUC ↑ 610%	(Simonson et al., 2004)
	Rifampin	Glyburide	AUC ↑ 125% (single dose IV)	(Zheng et al., 2009)
Intestinal OATP	Grapefruit juice	Fexofenadine	AUC ↓ 52%	(Glaeser et al., 2007)
	Grapefruit, orange and apple juice	Aliskiren	AUC ↓ 60%	(Tapaninen et al., 2010a; Tapaninen et al., 2010b)
OAT	NSAIDs	Methotrexate	Renal CL ↓ 20%	(Kremer and Hamilton, 1995)
	Probenecid	Acyclovir	AUC ↑ 40% ,Renal CL ↓ 32%	
OCT	Certirizine	Pilsicainide	Renal CL ↓ 41%	(Tsuruoka et al., 2006)
	Cimetidine	Metformin	AUC ↑ 50% ,Renal CL ↓ 27%	
ABC transporters				
P-gp	Ritonavir	Digoxin	AUC ↑ 86% ,Renal CL ↓ 35%	(Ding et al., 2004)
	Itraconazole	Fexofenadine	AUC ↑ 178%	
	Rifampin	Glyburide	AUC ↓ 63% (multi-dose PO)	(Zheng et al., 2009)
BCRP	Elacridar	Topotecan	AUC ↑ 140%	(Kruijtzer et al., 2002)
MRP	NSAIDs	Mycophenolate	↓ Enterohepatic circulation	(Fukuda et al., 2011)

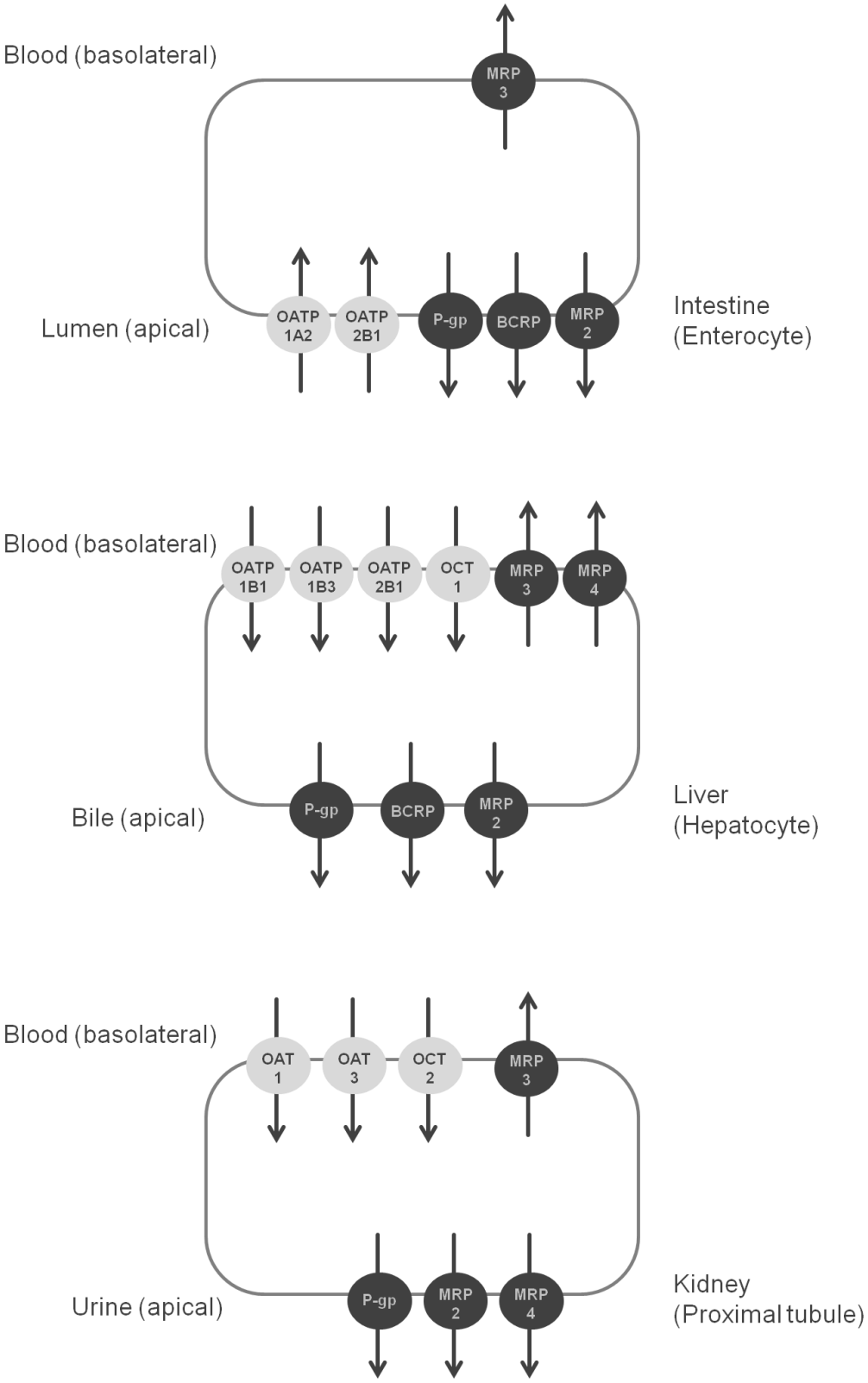

Figure 1-1. Localization of selected uptake and efflux transporters

CHAPTER 2
EFFECT OF OATP1B1 REDUCED-FUNCTION CARRIER STATUS ON ATORVASTATIN RESPONSE

Background

Hydroxymethylglutaryl-coenzyme A (HMG-CoA) reductase inhibitors, or statins, are widely used to treat dyslipidemia and reduce the risk of stroke and heart attack. While cardiovascular risk reduction is associated primarily with lowering low-density lipoprotein (LDL) concentrations, recent evidence indicates that anti-inflammatory effects also contribute to overall risk reduction (Albert et al., 2001; Waehre et al., 2004; Ridker et al., 2005; Zineh et al., 2006a; Zineh et al., 2008; Ridker et al., 2009; Carter, 2010; Lopez-Pedrera et al., 2012; Peña et al., 2012). Statins modulate inflammation in atherosclerosis through actions on pro-inflammatory cytokines, chemokines, and growth factors. Recently, the JUPITER trial demonstrated that rosuvastatin reduced cardiovascular events in individuals with low baseline LDL values who achieved goal concentrations of the inflammatory biomarker high-sensitivity C-reactive protein (hs-CRP) (Ridker et al., 2009).In our study, we sought to determine whether atorvastatin decreases the concentrations of the inflammatory markers thrombopoietin (TPO), monocyte chemotactic protein-1 (MCP-1), interleukin 1 receptor, type I (IL-1RA), and epithelial cell-derived neutrophil-activating peptide 78 (ENA-78).

These markers have been shown to be involved in inflammatory processes that contribute to cardiovascular disease. The cytokine IL-1 antagonist, IL-1RA, is released during inflammation, can be modulated by statins, and may protect against coronary artery disease (CAD) and acute coronary syndrome (ACS) (Waehre et al., 2004; Kwaijtaal et al., 2005; Rothenbacher et al., 2005; Wyss et al., 2010). MCP-1, a member of the CC chemokine family, is recognized as an angiogenic chemokine. MCP-1 acts as

a potent monocyte attractant and has been associated with atherosclerosis, acute coronary syndrome, and coronary artery disease (Aiello et al., 1999; de Lemos et al., 2003; Martinovic et al., 2005). The CXC chemokine ENA-78 attracts and activates neutrophils and has been implicated in various inflammatory conditions, including diabetes, heart failure, ischemic stroke, and acute coronary syndrome (Damas et al., 2000; Zaremba et al., 2006; Hasani Ranjbar et al., 2008; Zineh et al., 2008). TPO is a humoral growth factor that enhances platelet activation and adhesion and has been associated with unstable angina (Lupia et al., 2006). However, it is unknown how atorvastatin treatment alters these inflammatory markers acutely in a clinical setting.

Additionally, variability in inflammatory marker changes should be considered as the response to statin treatment is highly variable (Zineh, 2007). The variability in response may be explained in part through genetic variation in hepatic uptake transporters, which has the potential to affect both pharmacokinetics and pharmacodynamics of statins. Statins are subject to extensive first-pass metabolism and hepatic uptake. Hepatic uptake is facilitated by the hepatic organic anion-transporting polypeptides (OATPs), particularly OATP1B1, which is encoded by the gene *SLCO1B1*. Polymorphisms in *SLCO1B1* (e.g. 388A>G and 521T>C) have been shown to affect the disposition as well as drug response of statins and other OATP1B1 substrates (Niemi; Marzolini et al., 2004; Tachibana-Iimori et al., 2004; Thompson et al., 2005; Maeda et al., 2006; Takane et al., 2006; Zhang et al., 2007; Link et al., 2008; Maeda and Sugiyama, 2008; Kalliokoski and Niemi, 2009; Romaine et al., 2010).

In a pharmacokinetic study evaluating the 521T>C SNP, atorvastatin exposure, as measured by the area under the concentration-time curve (AUC), was 61% and 144%

greater in heterozygous and homozygous carriers of the variant allele, respectively (Pasanen et al., 2007). Likewise, the 388A>G and 521T>C SNPs have been shown to be associated with an attenuated statin response, in terms of changes in LDL, total cholesterol, and high-density lipoprotein (HDL) (Tachibana-Iimori et al., 2004; Thompson et al., 2005; Takane et al., 2006; Zhang et al., 2007). However, patients taking atorvastatin were only a small subset of a study evaluating the effect of the 521T>C SNP on statin response (Tachibana-Iimori et al., 2004). While OATP1B1 SNPs have been shown to have a substantial impact on atorvastatin exposure, little is known about how these SNPs may affect response to atorvastatin, particularly an anti-inflammatory response.

We hypothesized that atorvastatin would reduce inflammatory marker concentrations and that atorvastatin response would be mitigated (lipoprotein markers) or enhanced (inflammatory markers) in OATP1B1 reduced-function haplotype carriers as compared to the non-carriers. The objective of this study was to determine the effect of atorvastatin treatment as well as OATP1B1 reduced-function carrier status on lipoprotein (total cholesterol, low-density lipoprotein, high-density lipoprotein, and triglyceride) and inflammatory marker (high-sensitivity C-reactive protein (hs-CRP), thrombopoietin (TPO), monocyte chemotactic protein-1 (MCP-1), interleukin 1 receptor, type I (IL-1RA), and epithelial cell-derived neutrophil-activating peptide 78 (ENA-78)) concentrations in our study population of individuals without cardiovascular disease.

Methods

Study Population

The study population and protocol have been described previously (Zineh et al., 2006b). Briefly, individuals were eligible for participation if they were at least 18 years of

age and without known coronary disease, symptomatic carotid artery disease, peripheral vascular disease, abdominal aortic aneurysm, diabetes mellitus, dyslipidemia requiring treatment, or Framingham 10-year cardiovascular disease risk greater than 20%. Additionally, individuals were excluded for pregnancy, malignancy, liver transaminase levels greater than 2 times the upper limit of the laboratory reference range, active alcohol abuse, history of myositis, current treatment with systemic glucocorticoids or anti-inflammatory drugs, and previous treatment with any prescribed lipid-lowering therapy. All participants provided written informed consent; the study protocol was approved by the University of Florida Institutional Review Board.

Study Protocol

Individuals eligible for participation started a 2-week control-phase in which no atorvastatin was administered and then a treatment-phase in which they received atorvastatin 80 mg per day for 16 weeks. Study visits occurred every 4 weeks during the treatment-phase. At these study visits, the participants were counseled to maintain their current level of diet and exercise and blood was drawn for biochemical analysis. Blood samples were collected in the fasting state at baseline, 8-, and 16-weeks for low-density lipoprotein (LDL) and high-sensitivity C-reactive protein (hs-CRP) analyses in a clinical laboratory. Women of child-bearing potential had to use a reliable form of contraception throughout the study and were given a pregnancy test at each study visit. Blood for DNA isolation was collected from participants who consented to subsequent genetic analyses during the original study.

Inflammatory Marker Measurement

Blood hs-CRP concentrations were determined by biochemical analysis in the Shands Hospital clinical laboratory. Thrombopoietin (TPO), monocyte chemotactic

protein-1 (MCP-1), interleukin 1 receptor, type I (IL-1RA), and epithelial cell-derived neutrophil-activating peptide 78 (ENA-78) protein concentrations were measured in plasma using standard ELISA methods (R&D Systems Inc., Minneapolis, MN). To avoid diurnal variation in cytokine expression, blood samples were drawn between 7:00 am and 10:00 am. All samples were assayed in duplicate.

Genotyping

Genotypes of each subject were determined by polymerase chain reaction (PCR) with subsequent Pyrosequencing methods for the 388A>G (rs2306283) and 521T>C (rs4149056) single nucleotide polymorphisms (SNPs) within the *SLCO1B1* gene. The pyrosequencing PCR reaction contained approximately 50 ng (2µl) of template DNA, 10 pmol (1µl) each of forward and reverse primers, 8.5 µl H2O, and 12.5 µl GoGreen Taq™ Master Mix (Promega, Madison, WI, USA). The sequencing primers are listed in Table 2-1. PCR conditions were: 95°C for 2 min, followed by 45 cycles of 95°C for 30 s, 58°C for 30 s, and 72°C for 30 s, and a final extension at 72°C for 7 min. To detect polymorphisms, the PSQ 96A genotyping platform (Biotage AB, Uppsala, Sweden) was used for pyrosequencing. For sequencing reactions, between 4 and 7 µl of PCR product was used for sequencing reactions. All subject samples were assayed in duplicate.

Haplotype Assignments

Genotypes were used to determine reduced-function haplotype carrier status. *SLCO1B1* haplotypes were determined based upon the 388A>G and 521T>C SNPs, with no variants as *1a or wild type, 388A>G SNP as *1b, 521T>C SNP as *5, and both 388A>G and 521T>C SNPs as *15. Haplotype estimates were inferred using the Bayesian method based program PHASE (Stephens et al., 2001; Stephens and Scheet,

2005). For our analysis, we designated carriers of the *1a and *1b alleles as non-carriers and the *5 and *15 alleles as reduced-function carriers.

Statistical Analysis

Results are expressed as mean difference ± standard deviation. The data were analyzed using the program SAS for Windows version 9.3 (SAS Institute, Cary, NC). Inflammatory marker concentrations at baseline and after atorvastatin treatment were compared using the two-tailed paired t-test. The Hotelling's T-Square and the two-tailed unpaired t-test were used to compare atorvastatin's effect on inflammation between the OATP1B1 reduced-function carrier and non-carrier groups. Measurements from week 8 were carried forward for subjects missing week 16 measurements (n=9). Variances were compared by the F test. Differences were considered statistically significant if P was less than 0.05. Based upon the number of subjects in our study, it was estimated that we were able to detect an effect size of 0.68 standard deviations for lipoprotein and inflammatory marker concentrations between baseline and after treatment in the entire study population and between the OATP1B1 reduced-function carrier and non-carrier groups, respectively, with 80% power and a-level 5%.

Results

Study Population Characteristics

The study population included 81 individuals with a mean age of 31.2 (± 13) years. The study population consisted of 64% females, 73% Caucasians, 10% Hispanics, 7% Asians, 5% African-Americans, and 5% self-identified as "other race or ethnicity." Subjects were determined to be carriers or non-carriers of a reduced-function haplotype based upon their genotypes of the 388A>G and 521T>C SNPs in the *SLCO1B1* gene.

There were 26 subjects (32%) classified as reduced-function carriers and 55 subjects (68%) classified as non-carriers.

Atorvastatin Effects on Inflammation

We compared lipoprotein and inflammatory marker concentrations at baseline and after atorvastatin treatment (Table 2-2). As expected, we detected significant decreases in total cholesterol (34%), low-density lipoprotein (LDL) (55%), and triglyceride (27%) concentrations over the treatment period. In addition, we detected a significant decrease in serum thrombopoietin (TPO) (6%), however, there were no other significant changes in inflammatory marker concentrations.

Genetic Associations with Atorvastatin Response

We compared the atorvastatin response by change in lipoprotein and inflammatory markers concentrations between the OATP1B1 reduced-function haplotype carrier and non-carrier groups (Table 2-3). We found no significant differences in change of total cholesterol, LDL, HDL, or triglyceride concentrations. Total cholesterol decreased by 33% and 36% and LDL decreased by 54% and 56% in the non-carrier and carrier groups, respectively. Further, we did not detect any significant differences in change of high-sensitivity C-reactive protein (hs-CRP), epithelial cell-derived neutrophil-activating peptide 78 (ENA-78), monocyte chemotactic protein-1 (MCP-1), interleukin 1 receptor, type I (IL-1RA), or thrombopoietin (TPO) concentrations.

Discussion

In this study, we hypothesized that atorvastatin would reduce inflammatory marker concentrations and that haplotypes associated with reduced *SLCO1B1* function would alter atorvastatin response. In our study, we observed a 6% decrease in serum thrombopoietin (TPO) concentrations after atorvastatin treatment, but we were unable to

detect any other significant reductions in inflammatory marker concentrations. Further, OATP1B1 reduced-function haplotype carrier status did not alter atorvastatin-mediated changes in lipoproteins or inflammatory markers.

Previous studies have shown that statins exert pleiotropic effects, including modulating inflammation. The PRINCE study showed that pravastatin reduced concentrations of the inflammatory marker hs-CRP by 16.9% and 13.1% after 24 weeks of treatment in primary and secondary prevention groups, respectively (Albert et al., 2001). Recently, results of the JUPITER trial revealed significant risk reduction (65%) of myocardial infarction, stroke, unstable angina, arterial revascularization, and cardiovascular death in individuals taking rosuvastatin who achieved a dual LDL (<70 mg/dl) and hs-CRP (<2 mg/L) reduction goal (Ridker et al., 2009). Risk reduction attributed to hs-CRP reduction was determined to be independent of LDL reduction and greater risk reduction (79%) was observed for individuals who had hs-CRP levels less than 1 mg/L (Ridker et al., 2009). Likewise, dual LDL (<70 mg/dl) and hs-CRP (<2 mg/L) reduction was also compared between atorvastatin and pravastatin treatment in an analysis of the PROVE-IT TIMI-22 trial (Ridker et al., 2005). In this analysis, individuals who met both goals had a 28% lower risk of recurrent myocardial infarction and vascular death, and of those the majority received atorvastatin 80 mg daily. These studies have shown reductions in inflammation, as measured by the marker hs-CRP, in individuals receiving statin treatment for primary and secondary prevention. In addition to hs-CRP, other inflammatory markers are also of interest in statin treatment. We chose to measure the inflammatory markers thrombopoietin (TPO), monocyte chemotactic protein-1 (MCP-1), interleukin 1 receptor, type I (IL-1RA), and epithelial

cell-derived neutrophil-activating peptide 78 (ENA-78) as these markers may be modulated by statins and have been associated with cardiovascular disease (Aiello et al., 1999; Damas et al., 2000; de Lemos et al., 2003; Waehre et al., 2004; Kwaijtaal et al., 2005; Martinovic et al., 2005; Rothenbacher et al., 2005; Lupia et al., 2006; Zaremba et al., 2006; Zineh et al., 2006a; Hasani Ranjbar et al., 2008; Zineh et al., 2008; Wyss et al., 2010).

In our study, atorvastatin did not significantly reduce inflammatory marker concentrations, with the exception of TPO, in our population. Our population consisted of generally healthy individuals who likely did not have a high level of inflammation at baseline. Others studies that did show reductions in inflammatory marker concentrations did so in individuals who either had heart disease or were considered at risk for heart disease. In this study, subjects received high-dose atorvastatin (80 mg) for 16 weeks, which we believe would be a sufficient dose and duration to observe an effect. In addition, there was considerable variability in both baseline and after-treatment inflammatory marker concentrations in our study population and it could be that we were unable to detect smaller differences. Because of the high variability, we decided to investigate whether genetic variation could explain variability in atorvastatin-mediated changes in inflammatory maker concentrations.

Statins elicit lipid lowering effects by inhibiting HMG CoA reductase, an integral enzyme in cholesterol synthesis. The sites of action and metabolism for statins are within the hepatocytes. OATP1B1 facilitates hepatic uptake of statins and largely determines statin concentrations within the liver. It stands to reason that factors that affect the function of OATP1B1 would also affect statin concentrations due to altered

hepatic uptake. For instance, the 521T>C SNP within the *SLCO1B1* gene is associated with reduced OATP1B1 hepatic uptake; Pasanen et al. demonstrated that the area under the concentration-time curve (AUC) of atorvastatin was markedly higher in carriers of the 521T>C SNP as compared to non-carriers (Pasanen et al., 2007). Furthermore, the increase in AUC of atorvastatin in individuals homozygous for the 521T>C variant (CC) was more than two-fold higher as compared to the heterozygous (TC) individuals (Pasanen et al., 2007). The 521T>C SNP has also been implicated in atorvastatin drug interactions as being a determinant in the severity of the interaction (He et al., 2009). The effects of this SNP are not limited to atorvastatin as the pharmacokinetic profiles of other statins are also altered by *SLCO1B1* SNPs (Ho et al., 2007; Choi et al., 2008; Deng et al., 2008).

Considering the critical role of OATP1B1 in statin disposition and the evident effects of *SLCO1B1* SNPs on transport function, the effects of *SLCO1B1* SNPs on statin response is of utmost interest. Most recently, a genome-wide association study in the SEARCH trial evaluated approximately 300,000 markers in individuals with simvastatin-induced myopathy. The results implicated a single variant, the OATP1B1 521T>C SNP, and the investigators estimated over 60% of the myopathy cases were attributed to the 521C variant (Link et al., 2008). This finding was replicated in the Heart Protection Study, which also identified for simvastatin an association between the 521T>C SNP and myopathy as well as lipid-lowering (Link et al., 2008).

The effect of *SLCO1B1* SNPs on statin response as measured by lipid-lowering has been evaluated in other studies as well (Tachibana-Iimori et al., 2004; Thompson et al., 2005; Takane et al., 2006; Zhang et al., 2007). The 521T>C SNP alone and in

combination with the 388A>G SNP has been associated with decreased pravastatin efficacy in patients with hypercholesterolemia and coronary heart disease (Takane et al., 2006; Zhang et al., 2007). Atorvastatin was included in a small retrospective analysis in which the investigators found that subjects with the 521C variant experienced a diminished statin response (total cholesterol-lowering) as compared to subjects without the variant allele (Tachibana-Iimori et al., 2004). However, in this study, the majority of the subjects received either simvastatin or pravastatin, and only a small percentage (15%) of the subjects received atorvastatin (Tachibana-Iimori et al., 2004). In a larger analysis from the Atorvastatin Comparative Cholesterol Efficacy and Safety Study (ACCESS), 43 SNPs associated with statin response, including SNPs within the *SLCO1B1* gene, were evaluated in individuals receiving atorvastatin or one of four other statins (Thompson et al., 2005). In the ACCESS study, the OATP1B1 SNP 359+97C>A (rs4149036) was found to be associated with changes in triglyceride and the OATP1B1 SNPs 1748-97G>C (rs4149080) and 521T>C were associated with changes in HDL concentrations (Thompson et al., 2005), but neither was associated with LDL concentrations. However, the subjects were evaluated every 6 weeks over 24 weeks and statin doses were titrated until they achieved goal cholesterol levels, which may have masked poor responders. Due to limited data, there is still a lack in knowledge of how OATP1B1 SNPs affect atorvastatin response.

In our study, we evaluated *SLCO1B1* haplotypes based upon the 388A>G and 521T>C SNPs. For our analysis, we grouped the OATP1B1*1a and *1b haplotypes as non-carriers, and the *5 and *15 haplotypes as reduced-function carriers. We felt that it was appropriate to group the haplotypes as non-carriers and carriers of reduced-

function haplotype since the *1b is generally associated with OATP1B1 function comparable to the *1a or wild type haplotype, and the *5 and *15 haplotypes are both associated with reduced OATP1B1 function. Many in vitro studies have demonstrated comparable transport function between the OATP1B1*1a and *1b haplotypes (Tirona et al., 2001; Nozawa et al., 2002; Iwai et al., 2004; Kameyama et al., 2005; Ho et al., 2006), including similar transport of statins (Kameyama et al., 2005; Ho et al., 2006). Conversely, in vivo pharmacokinetic studies with pravastatin have suggested an increase in transport is associated with the *1b haplotype (Mwinyi et al., 2004; Maeda et al., 2006), but these studies were small and results from one of them only showed a trend toward decreased pravastatin AUC that was not statistically significant (Mwinyi et al., 2004).

We did not find an association between OATP1B1 reduced-function carrier status and atorvastatin response in our study. As the previous study protocol was not designed to collect blood samples for pharmacokinetic analysis, we were unable to determine if there were any differences in atorvastatin exposure based upon OATP1B1 haplotype status. However, based on previous studies a relevant difference in exposure could be expected. Since the OATP1B1 reduced-function 521T>C SNP has been shown to dramatically increase systemic atorvastatin exposure (AUC) (Pasanen et al., 2007), we expected to observe altered atorvastatin response in our study population.

However, we did not observe any effect of OATP1B1 reduced-function status on atorvastatin response, and there may be a few reasons for this. First, atorvastatin is transported by other transporters in addition to OATP1B1, including the hepatic uptake transporter, OATP2B1. In the presence of reduced-function OATP1B1, atorvastatin

uptake may be compensated for by other transporters. Also, we may have observed an altered response with a different statin, such as simvastatin, since OATP1B1 has shown substrate specific effects. Lastly, in our study population, there were only two individuals who were homozygous for 521T>C variant (*5/*5 haplotype). In a previous clinical pharmacokinetic study, Pasanen et al. detected a difference in simvastatin acid AUC between subjects homozygous for the 521T>C variant (CC) and heterozygous (TC) or wild type (TT) subjects, but no difference when the TC and TT subjects were compared (Pasanen et al., 2006). Differences in atorvastatin response may have been observed with more individuals homozygous for the 521T>C variant.

In conclusion, atorvastatin treatment did not significantly reduce inflammatory marker concentrations in our study of healthy individuals without cardiovascular disease. Further, reduced-function OATP1B1 haplotype carrier status did not have an effect on atorvastatin-mediated changes in lipoprotein and inflammatory marker concentrations.

Table 2-1. Polymerase chain reaction primers

Polymorphism	Primers (5' to 3')
SLCO1B1 c.388 A>G	
Forward	[BioTEG]TGGTGCAAATAAAGGGGAATA
Reverse	ATGTTGAATTTTCTGATGA
SLCO1B1 c.521 T>C	
Forward	[BioTEG]GGAATCTGGGTCATACATGTGG
Reverse	AAGCATATTACCCATGAAC

Table 2-2. Mean difference of lipoprotein and inflammatory marker concentrations from baseline

Measurement	Baseline	Treatment	Mean Difference	P value
Total cholesterol	181 ± 38.9	119 ± 26.0	-62.1 ± 28.8	<0.0001
LDL	101 ± 31.2	46.1 ± 19.7	-55.3 ± 23.3	<0.0001
HDL	60.8 ± 17.5	59.4 ± 16.8	-1.48 ± 10.6	0.2102
Triglycerides	96.3 ± 52.7	70.0 ± 42.4	-26.3 ± 33.9	<0.0001
hs-CRP	2.07 ± 3.07	1.75 ± 3.67	-0.14 ± 3.73	0.7311
ENA-78	1544 ± 1556	1433 ± 1581	-111 ± 690	0.1509
MCP-1	168 ± 68.5	159 ± 65.4	-9.45 ± 56.9	0.1385
IL-1RA	602 ± 400	610 ± 384	8.30 ± 229	0.7455
TPO	348 ± 159	328 ± 151	-20.5 ± 81.4	0.026

Data expressed as mean ± standard deviation

Table 2-3. Mean difference of lipoprotein and inflammatory marker concentrations from baseline in non-carriers and carriers of a reduced-function OATP1B1 haplotype

Measurement	Non-carriers (n=55)	Carriers (n=26)	P value
Total cholesterol	-60.4 ± 28.5	-65.6 ± 29.8	NS
LDL	-54.3 ± 23.7	-57.4 ± 22.7	NS
HDL	-0.24 ± 10.8	-4.12 ± 9.59	NS
Triglycerides	-28.9 ± 30.4	-20.8 ± 40.4	NS
hs-CRP	0.43 ± 4.00	-1.34 ± 2.80	NS
ENA-78	-99.1 ± 584	-137 ± 888	NS
MCP-1	-5.63 ± 45.3	-17.6 ± 76.2	NS
IL-1RA	18.3 ± 232	-12.8 ± 228	NS
TPO	-15.4 ± 83.8	-31.2 ± 76.5	NS

Data expressed as mean ± standard deviation

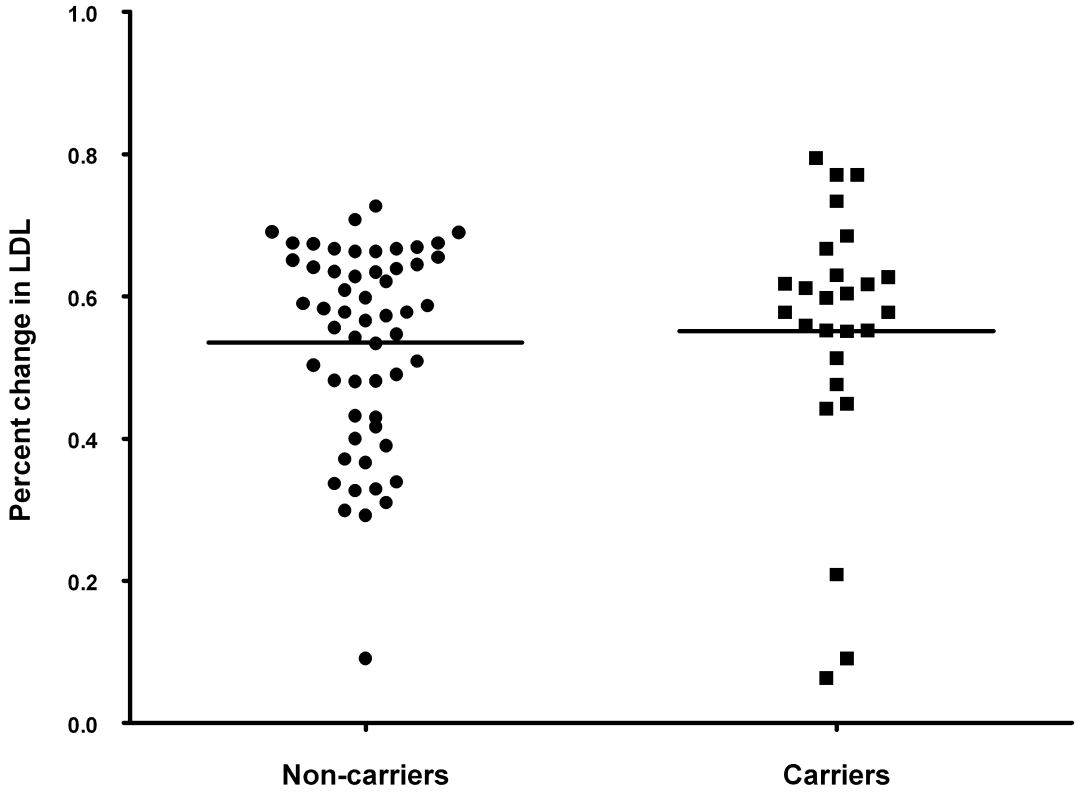

Figure 2-1. Percent reduction in LDL in non-carriers and carriers of a reduced-function OATP1B1 haplotype

CHAPTER 3
VALIDATION AND APPLICATION OF A LIQUID CHROMATOGRAPHY-TANDEM MASS SPECTROMETRIC METHOD FOR QUANTIFICATION OF THE DRUG TRANSPORT PROBE FEXOFENADINE IN HUMAN PLASMA USING 96-WELL FILTER PLATES[1]

Background

Fexofenadine is a histamine H1-receptor antagonist used therapeutically for the treatment of allergic rhinitis and chronic idiopathic urticaria (Devillier et al., 2008); it is given orally in doses ranging from 30 to 180 mg/day. Fexofenadine is predominantly eliminated unchanged in bile (80%) and urine (11%) (Devillier et al., 2008); approximately 5% is metabolized forming methyl ester (3.6%) and azacyclonol (1.5%) metabolites (Lippert et al., 1995). The major determinants of fexofenadine absorption and elimination are the activity of drug transporters located in the intestine and liver (Devillier et al., 2008). Specifically, fexofenadine is a substrate of the transporters P-glycoprotein (P-gp) and the organic anion-transporting polypeptides (OATP) OATP1A2 and OATP1B3 (Cvetkovic et al., 1999; Shimizu et al., 2005; Devillier et al., 2008; Matsushima et al., 2008a). The absorption of fexofenadine in the intestine is limited by the efflux transporter P-gp but is enhanced by the uptake transporter OATP1A2 (Cvetkovic et al., 1999), while the elimination of fexofenadine in the liver is dependent on the hepatic uptake transporter OATP1B3 (Shimizu et al., 2005). Thus, fexofenadine is used as a pharmacologic probe of human drug transporters to characterize transporter activity in interaction studies with concomitant drugs, foods, or herbal products (Wang et al., 2002; Dresser et al., 2005; Kharasch et al., 2005; Yasui-Furukori

[1] Reprinted with permission from Stanton ML, Joy MS, and Frye RF (2010) Validation and application of a liquid chromatography-tandem mass spectrometric method for quantification of the drug transport probe fexofenadine in human plasma using 96-well filter plates. *J Chromatogr B Analyt Technol Biomed Life Sci* **878:**497-501.

et al., 2005; Shimizu et al., 2006; van Heeswijk et al., 2006; Bailey et al., 2007; Glaeser et al., 2007; Robertson et al., 2008) and to evaluate the effects of genetic variation or disease state on transporter activity and function (Yi et al., 2004; Niemi et al., 2005b; Shon et al., 2005). As drug transport is increasingly recognized as a critical pathway in the disposition of many drugs, there is a need for simple analytical methods for probes such as fexofenadine to facilitate evaluations of drug transporter activity.

Several HPLC methods for determination of fexofenadine in biologic fluids have been reported (Coutant et al., 1991; Hofmann et al., 2002; Naidong et al., 2002; Fu et al., 2004; Uno et al., 2004; Nirogi et al., 2006; Emara et al., 2007; Isleyen et al., 2007; Miura et al., 2007b; Nirogi et al., 2007; Yamane et al., 2007; Bharathi et al., 2008; Pathak et al., 2008; Guo et al., 2009). A few methods use ultraviolet (Emara et al., 2007; Miura et al., 2007b) or fluorescence detection (Coutant et al., 1991; Uno et al., 2004; Pathak et al., 2008), but most methods for determination of fexofenadine in human plasma are based on HPLC with mass spectrometric detection (Hofmann et al., 2002; Isleyen et al., 2007) and tandem mass spectrometric detection (Naidong et al., 2002; Fu et al., 2004; Nirogi et al., 2006; Nirogi et al., 2007; Yamane et al., 2007; Bharathi et al., 2008) because of better sensitivity and selectivity. Sample processing for almost all of the methods reported use costly solid phase extraction (SPE) with C18 cartridges (Hofmann et al., 2002) or Oasis™ HLB cartridges (Naidong et al., 2002; Fu et al., 2004; Nirogi et al., 2006; Nirogi et al., 2007; Yamane et al., 2007; Bharathi et al., 2008). The typical range of quantification for the MS-based methods is 1-500 ng/ml and the run times range from 2 to 10 min. Most of the methods reported use structurally related compounds for the internal standard (e.g., diphenhydramine, loratadine,

terfenadine) (Hofmann et al., 2002; Naidong et al., 2002; Nirogi et al., 2006; Isleyen et al., 2007; Miura et al., 2007b; Nirogi et al., 2007; Yamane et al., 2007; Bharathi et al., 2008); only the method by Fu et al., (Fu et al., 2004), uses a deuterated internal standard (fexofenadine-d6), but that method requires 0.5 ml of plasma for processing by SPE. Protein precipitation in microcentrifuge tubes was used in a method reported recently by Guo et al., (Guo et al., 2009), but the oral hypoglycemic drug glipizide was used as the internal standard.

Herein, we present the validation of a sensitive method for fexofenadine determination in human plasma by liquid chromatography-tandem mass spectrometry. The method has several advantages including rapid sample processing based on protein precipitation and filtration in a 96-well plate format, a deuterated internal standard (fexofenadine-d6), a small sample volume requirement (100 µl human plasma) and a total run time of 2 min with isocratic elution. The method is suitable for determination of fexofenadine concentrations in clinical pharmacokinetic studies.

Experimental

Chemicals and Reagents

Fexofenadine (>98% chemical purity) was purchased from Sigma-Aldrich Co. (St. Louis, MO, U.S.A) and the deuterated internal standard fexofenadine-d6 (>98% chemical and >99% isotopic purity) was purchased from Toronto Research Chemicals Inc. (North York, ON, Canada). Chemical structures are shown in Figure 3-1. Acetonitrile and methanol, HPLC grade, and formic acid, analytical grade were purchased from VWR International, LLC (West Chester, PA, USA). Human EDTA plasma was obtained from the UF & Shands Hospital blood bank (Gainesville, FL, USA); plasma was screened for the presence of fexofenadine prior to use. HPLC grade

deionized water was obtained from a Barnstead Nanopure Diamond UV Ultrapure Water System (Dubuque, IA, USA).

Instrumentation and Chromatographic Conditions

The LC-MS/MS system included a Surveyor HPLC autosampler, Surveyor MS quaternary pump and a TSQ Quantum Discovery triple quadrupole mass spectrometer (Thermo Scientific, San Jose, CA, USA). The TSQ Quantum mass spectrometer was equipped with an electrospray ion source (ESI) with the ESI source spray set orthogonal to the ion transfer capillary tube. The autosampler temperature was maintained at 10°C. The analytical column was a Gemini C18, 50×2.0 mm, 5 µm (Phenomenex, Torrance, CA, USA). The mobile phase consisted of deionized water and methanol (35:65, v/v) that contained 0.1% formic acid and 5mM ammonium acetate and was pumped at a flow rate of 0.2 ml/min. The mobile phase was degassed and filtered through a 0.22µm Nylon 66 membrane prior to use. The MS/MS conditions were optimized by using an infusion system with a mixing tee. Fexofenadine (1 µg/ml at 5 µl/min) was infused in one line of a mixing tee while mobile phase was delivered at 0.2 ml/min in the other line. For quantification, the TSQ Quantum was operated in high resolution single reaction monitoring mode (H-SRM). The ESI was operated in the positive mode at a spray voltage of 4.6kV and source CID -10V with a heated capillary temperature of 375^{o}C. Nitrogen was used as the sheath and auxiliary gas and the flow rates were set to 35 and 10 units (arbitrary), respectively. The argon collision gas pressure was set to 1.5 mTorr. The collision energy was -41 eV for fexofenadine and fexofenadine-d6 (internal standard). Fexofenadine was monitored at *m/z* 502.3 $\rightarrow$ 171.0 and fexofenadine-d6 at *m/z* 508.3 $\rightarrow$ 177.0.The instrument was operated in enhanced (high) resolution with

peak width (FWHM) set to 0.2 *m/z* at Q1 and to 0.7 *m/z* at Q3. The scan time was 300 ms for each transition. SRM data were acquired and processed using ThermoFinnigan XCalibur® software version 1.4, service release 1 (Thermo Scientific, San Jose, CA, USA).

Standard Preparation

Fexofenadine stock solutions were prepared in methanol at concentrations of 1, 10 and 100 µg/ml. Dilutions of these stock solutions were used to prepare calibration standards and quality control (QC) samples. The stock solution for the internal standard was prepared by dissolving fexofenadine-d6 in methanol at a concentration of 1 µg/ml and then further diluted in acetonitrile to a concentration of 35 ng/ml. These stock solutions were stored at -20°C. Calibration standards were prepared at concentrations of 1, 2, 5, 25, 50, 100, 250 and 500 ng/ml by spiking blank human plasma with varying quantities of the standard solutions (1, 10 or 100 µg/ml). These standard solutions were also used to prepare blank human plasma at concentrations of 10, 150 and 400 ng/ml for the QC samples. Standards and quality control samples were stored at -20°C until analysis.

Sample Preparation

Acetonitrile (300 µl) containing the internal standard (10.5 ng) and then plasma (100 µl) were pipetted into a 96-well Captiva™ filter plate (Varian Inc., Palo Alto, CA, USA). The filter plate was mixed briefly and then inverted for 5 minutes at room temperature. Next, the filter plate was fitted with a vacuum collar and 1 ml collection plate before filtration by vacuum pressure. The resulting filtrate was diluted with 400 µl water and injected into the HPLC system (10 µl).

Calibration and Linearity

Calibration standards over the concentration range of 1-500 ng/ml were analyzed in duplicate for three runs; the lowest standard was analyzed in triplicate. Back-calculated concentration values for each standard were considered acceptable if both the percent relative standard deviation (R.S.D.%) and the relative error (RE%) were within 15%; the lower limit of quantification (LLOQ) was acceptable if the R.S.D.% and R.E.% were within ±20%.

Precision and Accuracy

The intra- and inter-run precision (R.S.D.%) and accuracy (R.E.%) of the assay were determined by analyzing QC samples at concentrations of 10, 150 and 400 ng/ml for three runs. Six of each QC level were analyzed for two separate runs and twelve of each QC level were analyzed for one run (n=24). The calculated mean concentration relative to the spiked concentration was used to express accuracy as the relative error (R.E.%). Means, standard deviations and R.S.D.% were calculated from the QC values and used to estimate the intra- and inter-run precision. Dilution integrity was determined by processing six replicates of a dilution QC (1,000 ng/ml) after a 10-fold dilution. The mean accuracy was expressed as R.E.%.

Selectivity and Stability

Selectivity was evaluated by processing and analyzing blank plasma samples obtained from six different sources. Carry-over was evaluated by injections of mobile phase placed in several wells of the analysis set. The autosampler stability of processed samples was evaluated by analyzing QC samples immediately and 24 hours after processing. After the first analysis, the QC samples were stored in the autosampler at 10°C for at least 24 hours and then re-analyzed. The measured concentrations from

both analyses were then compared to determine any differences due to the storage conditions. The stability after freeze and thaw was evaluated with low and high-concentration QC samples, which were subjected to three freeze-thaw cycles prior to processing. The effects were measured by the concentrations of each QC relative to a newly processed reference sample.

Matrix Effects and Extraction Efficiency

The potential for matrix effects (suppression or enhancement of ionization) was evaluated qualitatively by standard post-column infusion experiments (King et al., 2000). Processed blank plasma samples from six independent sources were injected during a constant post-column infusion of fexofenadine. In addition, to determine the presence of matrix effects quantitatively, the response obtained from plasma samples (n=6) spiked after processing and mobile phase spiked with an equivalent amount of fexofenadine were compared. Responses obtained from the spiked fexofenadine solution were defined as 100%. Extraction efficiency at low and high QC concentrations (10 and 400 ng/ml) was determined by comparing fexofenadine response in plasma samples (n=6) spiked before and after extraction, which was defined as 100% recovery.

Application to Plasma Sampling

Fexofenadine pharmacokinetics were evaluated in a research volunteer with renal manifestations of systemic lupus erythematosus. The subject granted written informed consent and the study was approved by the Committee on the Protection of Human Subjects at the University of North Carolina. The study sought to evaluate fexofenadine as a P-glycoprotein probe substrate because glucocorticoids are substrates of this protein and are utilized in combination therapies for lupus nephritis. The subject had been receiving prednisone 60 mg daily for 90 days prior to the pharmacokinetics study.

The subject received intravenous cyclophosphamide at the time of fexofenadine administration. The subject was receiving aspirin which is a known inducer of P-glycoprotein; no known inhibitors were prescribed. Fexofenadine 60 mg (Allegra®, Aventis Pharmaceuticals Inc., Bridgewater, NJ, USA) was administered orally with 8 ounces of water. Plasma was collected over 72 hours and stored at -70oC until analyzed.

Results and Discussion

Chromatography

Representative extracted ion chromatograms of plasma samples are depicted in Fig. 2; Fig. 2A shows a blank plasma sample and Fig. 2B is a plasma sample spiked with fexofenadine at the LLOQ (1 ng/ml). Fig. 2C depicts a plasma sample obtained from a study subject after a single dose of fexofenadine 60 mg. A plasma sample spiked with the internal standard fexofenadine-d6 is shown in Fig. 2D. The retention time for fexofenadine and fexofenadine-d6 was 1.2 minutes.

Calibration and Linearity

The calibration curve was linear over a concentration range of 1-500 ng/ml using weighted (1/y2) linear regression, which was determined to be the best fit. Duplicate calibration curves were analyzed for three runs and the mean calibration curve equation was $y = 0.004069x \pm 0.000071 - 0.000867 \pm 0.000141$. The correlation coefficient (r^2) was greater than 0.99.

Precision and Accuracy

The intra- and inter-run accuracy (within ± 8%) and precision (within ± 4.3%) of the back calculated concentrations for the QC samples demonstrated an accurate and reproducible method (Table 2-1). Dilution integrity was determined by processing six

replicates of the dilution QC (1,000 ng/ml) after a 10-fold dilution. The accuracy and reproducibility was found to be acceptable with a mean accuracy of 0.4% (R.E.%) and precision of 3.9% (R.S.D.%).

Selectivity and Stability

No interfering peaks were observed for fexofenadine or fexofenadine-d6 in the processed plasma samples from six different sources and there was no evidence of carry-over. Autosampler stability was determined by comparing results for samples that were analyzed immediately and 24 hours after processing. The samples analyzed 24 hours after processing were stored in the autosampler at 10°C and the concentrations found did not deviate by more than 10%. The effect of freeze and thaw was evaluated in QC samples subjected to three freeze-thaw cycles and no degradation of fexofenadine was observed (i.e., less than a 10% difference in measured concentration).

Matrix Effects and Extraction Efficiency

Recovery of fexofenadine at low and high QC concentrations was measured by comparing the response ratios of plasma samples that were spiked before and after processing. The response in samples spiked after processing were considered to be 100%. We determined the recovery for fexofenadine to be 93.6 ± 6.5% at low and 95.3 ± 10.3% at high concentrations. In addition, there was no evidence of a matrix effect as there was < 10% difference in the fexofenadine response. The post-column infusion experiment supported a lack of matrix effect.

Application to Plasma Sampling

This method was used to support a fexofenadine pharmacokinetic study in which fexofenadine was used a probe for transporter activity in patients with glomerulonephritis. The concentration-time profile for a study subject after

administration of fexofenadine 60 mg as a single oral dose is shown in Fig. 3. The concentration values were consistent with values reported in the literature (Devillier et al., 2008). The method was shown to be suitable for pharmacokinetic studies of fexofenadine in human subjects.

Incurred Sample Reproducibility

To confirm assay accuracy and precision, we reanalyzed 20 incurred plasma samples using the same procedures as the initial analysis. Percentage differences in concentration were calculated and used to determine the average fold change or mean-ratio (MR), ratio limits (RLs), and limits of agreement (LsA). Statistical analysis of the results showed a MR of 1.01, RLs of 0.99-1.03 (acceptance range, 0.83-1.20), and LsA of 0.93-1.11 (acceptance range, 0.83-1.20). The acceptance ranges were met for both the RLs and LsA, demonstrating assay reproducibility.

Summary

We have validated a rapid, sensitive, selective, and reproducible LC-MS/MS method for determination of fexofenadine in human plasma. The method uses protein precipitation and filtration in a 96-well format, which from start to finish takes less than an hour to process manually. The rapid sample processing combined with the total chromatographic run time of 2 min. facilitates multiple runs per day for high throughput applications. The method is currently being used to support clinical pharmacokinetic studies with the drug transport probe fexofenadine.

Table 3-1. Intra- and inter-run precision (R.S.D.%) and accuracy (R.E.%) for fexofenadine quality control samples in human plasma.

Concentration (ng/ml)		Precision (R.S.D.%)	Accuracy (R.E.%)
Nominal	Found (mean ± SD)		
Intra-run (N=12)			
1 (LLOQ QC)	1.10 ± 0.22	20.0	10.0
10	10.45 ± 0.45	4.3	4.5
150	158.3 ± 2.0	1.2	5.5
400	429.4 ± 7.3	1.7	7.3
Inter-run (N=24)			
1 (LLOQ QC)	1.09 ± 0.21	19.7	8.9
10	10.41 ± 0.41	3.9	4.1
150	156.6 ± 2.8	1.8	4.4
400	431.9 ± 7.8	1.8	8.0

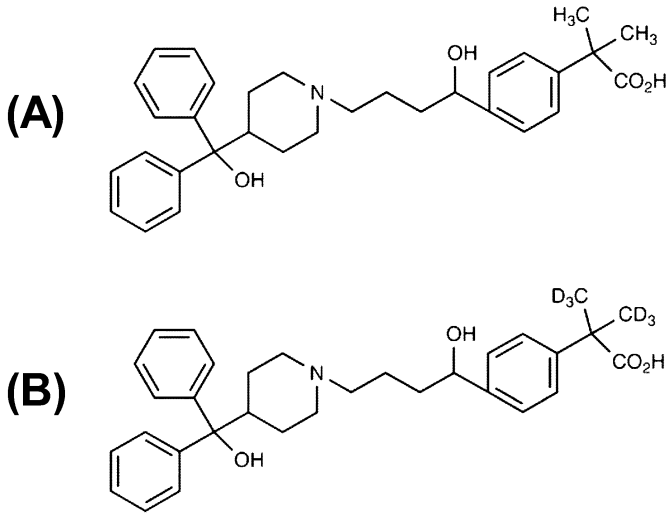

Figure 3-1. Chemical structures of (A) fexofenadine and (B) fexofenadine-d6 (internal standard)

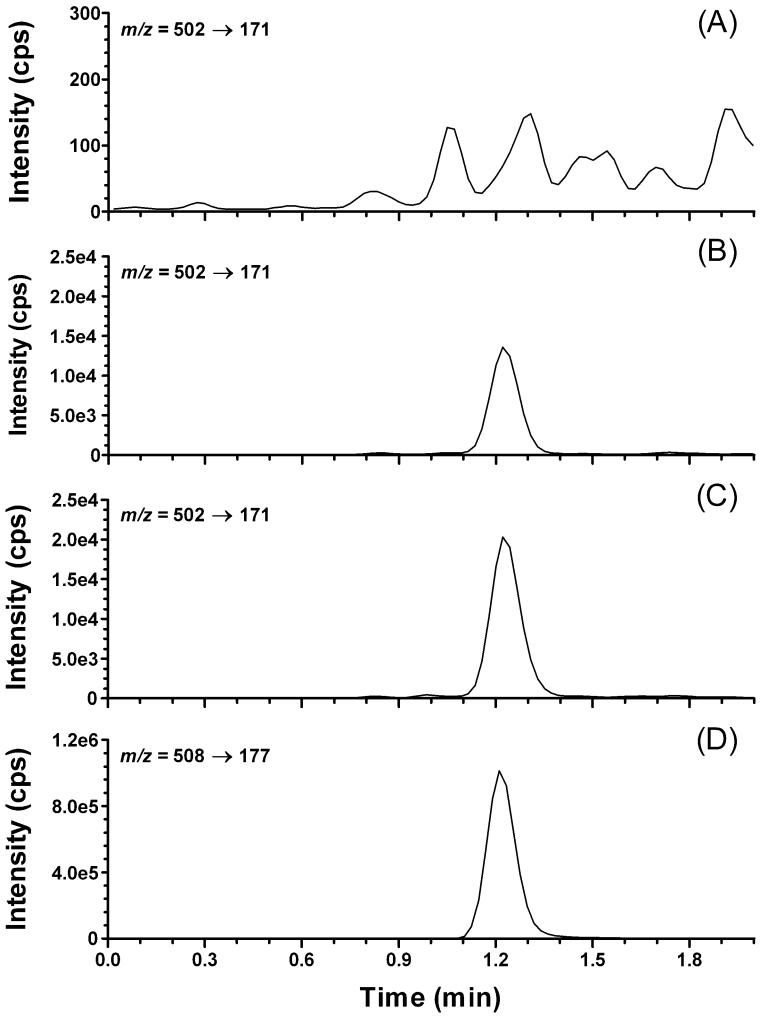

Figure 3-2. Representative extracted ion chromatograms of: (A) blank plasma (B) fexofenadine lower limit of quantitation (LLOQ; 1.00 ng/ml); (C) plasma sample from a subject obtained 48 hours after oral administration of fexofenadine 60 mg (concentration = 1.62 ng/ml); and (D) fexofenadine-d6 (ISTD)

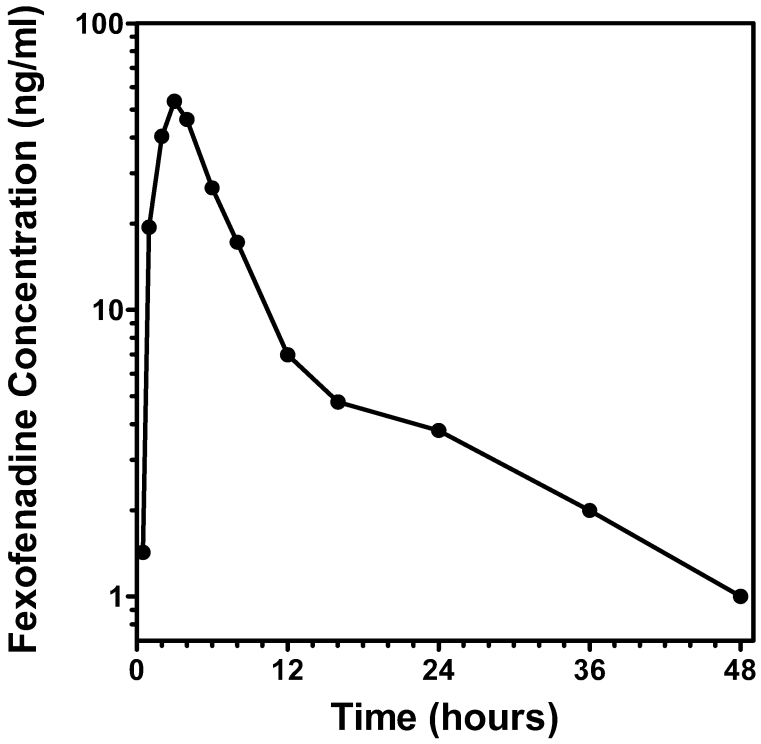

Figure 3-3. Concentration-time profile for a study subject administered single oral dose of fexofenadine (60 mg)

CHAPTER 4
EFFECTS OF GREEN TEA EXTRACT ON FEXOFENADINE PHARMACOKINETICS

Background

Drug transport plays a major role in the elimination of many drugs and is an important pathway of drug clearance (Giacomini et al., 2010). A key family of drug transporters is the organic anion-transporting polypeptide (OATP) family, which facilitate the uptake (absorption and/or elimination) of a large variety of drug substrates. Recognition of the important role of OATP-mediated transport in drug disposition is increasing. Many drug interactions thought to be mediated by OATPs have been reported (Han, 2011). The antihistamine drug fexofenadine is used as an in vivo probe for drug transporter activity since it is minimally metabolized (approximately 5%) (Lippert et al., 1995) and predominantly transported by intestinal OATP1A2 and hepatic OATP1B3 (Cvetkovic et al., 1999; Shimizu et al., 2005). Fexofenadine has been used to characterize OATP-mediated uptake in the presence of disease (Nolin et al., 2009), genetic variation (Akamine et al., 2010; Imanaga et al., 2011), and concomitant drug use (Yasui-Furukori et al., 2005; Matsushima et al., 2008b; Yamada et al., 2009).

Herb-drug and food-drug interactions are becoming increasingly important, as it is now estimated that up to 38% of Americans use complementary and alternative medicines (Su and Li, 2011) with up to 25% specifically using herbs or dietary supplements (Wu et al., 2011). Likewise, OATP-mediated food and herb-drug interactions have been reported (Dresser et al., 2005; Fuchikami et al., 2006; Fan et al., 2008; Mandery et al., 2010). Green tea extract is an herbal product commonly used for its purported antioxidant effects and health benefits. The effects of green tea extract on drug metabolism enzymes have been investigated in preclinical and clinical studies,

however, little is known about the effects of green tea extract on drug transporters (Donovan et al., 2004; Nishikawa et al., 2004; Chow et al., 2006; Netsch et al., 2006; Mohamed et al., 2010). In an *in vitro* model, green tea extract or more specifically epigallocatechin gallate (EGCG), the primary constituent in green tea extract, was reported to potently inhibit OATP2B1, which is expressed in the intestines and liver (Fuchikami et al., 2006). Moreover, in a recent report evaluating the effects of EGCG on cells expressing OATPs, EGCG was shown to inhibit OATP1B1- ,OATP2B1-, and OATP1A2-mediated uptake, while the effect of EGCG on OATP1B3-mediated uptake was substrate dependent as both stimulation and inhibition were observed (Roth et al., 2009). Based on these results, EGCG appears to have predominantly inhibitory effects on OATP-mediated transport. However, to our knowledge, these effects have not been investigated in a clinical study.

The aim of this study was to determine the effect of green tea extract on fexofenadine pharmacokinetics in order to characterize in vivo the effects of green tea extract on OATP-mediated uptake. We investigated the effect of single dose green tea extract (EGCG 800 mg) on fexofenadine pharmacokinetics in healthy volunteers.

Methods

Study Participants

Eight healthy volunteers (four males and four females) who were 18 years of age or older were enrolled in this study after giving written informed consent. The mean (± standard deviation) age and body mass index of study participants were 30.9 (±16.2) years (range 20-58 years) and 25.0 (±2.2 kg/m^2) (range 22.4-29.2 kg/m^2), respectively. Each study participant was deemed to be healthy by physical exam, routine laboratory

tests, and review of their medical histories. This study was approved by the University of Florida Institutional Review Board.

Study Design

This was a randomized open-label crossover study consisting of two phases, a control phase and the EGCG phase. The order of the control and EGCG phases for each of the participants were randomly assigned using SAS version 9.2 (SAS Institute, Cary, NC). The phases were separated by 7 to 21 days. For each of the two study visits, participants were admitted to the Shands Clinical Research Center (CRC) at 8:00 PM the evening prior to the dosing day. During the study visits, participants received a single oral dose of fexofenadine hydrochloride 60 mg either alone or after a single oral dose of green tea extract (EGCG 800 mg). For this study, we used EGCG Green Tea Extract (393 mg EGCG per dose) by Relentless Improvement LLC (Reno, NV) from a single lot. This product has been used previously in studies sponsored by the National Center for Complementary and Alternative Medicine (NCCAM) and was approved for use through their product integrity review committee. Fexofenadine was administered at 9:00AM for each study visit and green tea extract was administered at 8:00 AM during the green tea phase study visit. The participants received light snacks at 11:00 PM the evening of admission, 7:00 AM and 11:00 AM on the dosing day and were permitted to eat full meals after 1:00 PM and for the remainder of their study visit. Standardized meals and beverages were prepared and provided by the CRC Metabolic Kitchen. Study participants were asked to abstain from alcohol, fruit juices, herbal teas and supplements, and over-the-counter medications prior to and during the study period.

Plasma Collection and Determination of Fexofenadine and EGCG

Venous blood samples (7 ml) were drawn into EDTA blood collection tubes before and at 0.5, 1, 2, 3, 4, 5, 6, 9, 12, 15, and 24 hours after administration of fexofenadine. The blood samples were stored on ice and plasma was separated with 30 minutes of collection and then stored at -80°C until analyzed.

Fexofenadine plasma concentrations were determined by a sensitive and specific liquid chromatography tandem mass spectrometry method developed in our laboratory (Stanton et al., 2010). The limit of quantitation (LOQ) for fexofenadine is 1.0 ng/mL using 100 µl plasma and the within-run and between-run precision is less than 5%. Plasma samples from the EGCG phase did not have any interfering peaks in the fexofenadine assay.

Pharmacokinetic Analysis

Fexofenadine pharmacokinetic parameter values were estimated from the plasma concentration-time data using standard noncompartmental methods (WinNonlin, PharSight Corp., Mountain View, CA, USA). The maximum plasma concentrations (C_{max}), the time C_{max} occurs (t_{max}), and the last plasma concentration measured, or the 24-hour concentration (C_{24}), were obtained directly from the individual plasma concentration-time profiles. The terminal elimination rate constant (λ_z) was estimated by linear regression of the terminal phase of the logarithmic plasma-concentration time curve. Terminal elimination half-life ($t_{½}$) was calculated by dividing 0.693 by λ_z. Area under the plasma concentration-time curve from 0 to 24 hours (AUC_{0-24}) was calculated with the linear-log trapezoidal rule. The area under the plasma concentration-time curve from 0 to infinity ($AUC_{0-\infty}$) was calculated as $AUC_{0-24} + C_{24}/\lambda_z$. The apparent oral

clearance (CL/F) was calculated from the equation CL/F = Dose/AUC and the apparent volume of distribution at steady state (V_{ss}) was calculated from the equation V_{ss} = CL × MRT (mean residence time).

Statistical Analysis

The results are expressed as mean ± standard deviation and mean differences between the control and green tea phases. A two sample method for crossover studies was used to compare the fexofenadine pharmacokinetics between the two phases. Two-sided 95% confidence intervals were also computed. The sample size of n=8 allowed for detection of a difference of 1.17 SD in the raw log scale measurement for AUC at 80% power and α level 5%. Differences were considered statistically significant if p was less than 0.05. Linear regression was used to rule-out an order effect. Data was analyzed with SAS version 9.3 (SAS Institute, Cary, NC).

Results

Plasma Concentrations and Pharmacokinetics of Fexofenadine

Fexofenadine pharmacokinetic parameters following a single oral dose of 60 mg of fexofenadine in both the control and green tea phases are summarized in Table 4-1 and mean plasma fexofenadine concentration-time profiles are shown in Figure 4-1. Figures 4-2 and 4-3 depict fexofenadine $AUC_{0-\infty}$ and C_{max} values, respectively. The mean plasma concentrations of fexofenadine in the green tea phase were lower than those in the control phase (Figure 4-1). Green tea extract (EGCG 800 mg) significantly decreased fexofenadine mean $AUC_{0-\infty}$ from 872 to 294 ug·h/L (p = 0.0058) and mean C_{max} from 146 to 50.2 ug/L (p = 0.0154) and increased its CL/F (p = 0.0086) and V_{ss} (p =

0.0158) as compared with the control phase (Table 4-1). There were no significant differences in $t_{max,}$ or $t_{½}$ between the control and green tea phases (Table 4-1).

Discussion

In this study, we investigated the effect of green tea extract (EGCG 800 mg) on the pharmacokinetics of the OATP probe fexofenadine. To our knowledge, this is the first time the in vivo effects of EGCG on OATP-mediated transport have been characterized. The results demonstrate that coadministration of green tea extract altered the disposition of fexofenadine. In this study, green tea extract significantly decreased fexofenadine $AUC_{0-\infty}$ by 66% and C_{max} by 65% with relative increases in CL/F and V_{ss}. There were no significant changes in elimination rate or half-life. These results indicate that green tea extract decreases the bioavailability of fexofenadine, most likely through inhibition of OATP-mediated intestinal uptake.

Other drug interaction studies have shown similar results to our study (reduced fexofenadine bioavailability) when intestinal OATP1A2 uptake is inhibited (Dresser et al., 2005; Qiang et al., 2009). In an in vitro experiment, grapefruit and orange juice were shown to potently inhibit OATP1A2-mediated uptake of fexofenadine (Dresser et al., 2002). The investigators then evaluated this interaction in a healthy volunteer study and found that the fruit juices markedly reduced fexofenadine bioavailability with decreases in AUC and C_{max} of 30% and 40%, respectively, and did not alter half-life (Dresser et al., 2002). Further, fruit juices have similar effects on the disposition of aliskiren and this interaction is also thought to be mediated by intestinal inhibition of OATP1A2 and perhaps OATP2B1 (Tapaninen et al., 2010a; Tapaninen et al., 2010b; Rebello et al., 2011).

EGCG has been shown to inhibit both OATP1A2 and OATP2B1-mediated uptake in vitro (Roth et al., 2009). Additionally, EGCG was also shown to be a substrate of OATP1A2 (Roth et al., 2009). Some studies have shown that fexofenadine is a substrate for OATP2B1 uptake (Nozawa et al., 2004; Ming et al., 2011), however other groups have been unable to demonstrate significant uptake of fexofenadine by OATP2B1 and OATP1A2 appears to be the major determinant of fexofenadine uptake in the intestine (Dresser et al., 2005; Shimizu et al., 2005; Glaeser et al., 2007). If fexofenadine is a substrate for OATP2B1-mediated uptake in the intestine and if EGCG inhibits OATP2B1 in vivo, then we would still expect reduced fexofenadine concentrations, which is consistent with our results.

EGCG has also been shown to inhibit P-gp efflux in an in vitro study (Jodoin et al., 2002). Fexofenadine is a substrate for P-gp efflux (Cvetkovic et al., 1999) and drug interactions involving changes in P-gp transport have been reported (Hamman et al., 2001; Shimizu et al., 2006; Kim et al., 2009; Yamada et al., 2009). However, when P-gp efflux is inhibited, fexofenadine concentrations are significantly increased, not decreased. On the other hand when drug interactions result in P-gp induction, fexofenadine concentrations, AUC, and C_{max} are reduced as we observed in our study. However, EGCG has only been shown to inhibit not induce P-gp (Jodoin et al., 2002) and the current study involved only single dose EGCG administration.

The effects of EGCG on metabolic enzymes have also been studied. There have been no clinically significant effects of EGCG on major cytochrome P450 (CYP) enzymes (CYP3A4, CYP2D6, CYP2C9, and CYP1A2) (Donovan et al., 2004; Chow et al., 2006), but EGCG has been shown to inhibit the UDP glucuronosyltransferase

enzyme UGT1A1 (Mohamed et al., 2010). Yet, fexofenadine is predominantly eliminated unchanged with minimal metabolism (approximately 5%), so the interaction we observed cannot be explained by effects on metabolic enzymes.

Based upon the in vitro evidence of EGCG inhibition of OATP-mediated uptake and our results, we believe that green tea extract reduces fexofenadine bioavailability by inhibition of intestinal OATP uptake, most likely due to OATP1A2 inhibition. Although we did not observe any changes in half-life, we cannot rule out the possibility that green tea extract also inhibits hepatic OATP uptake in vivo as we only used oral fexofenadine in our study. The results of our study have considerable clinical implications as the disposition of many drugs is determined by OATP uptake. OATP1A2 is known to transport statins, antihypertensive drugs, antivirals, antibiotics, the anticancer drug methotrexate, and was recently identified to transport imatinib, a drug used to treat a number of different cancers (Kalliokoski and Niemi, 2009). The OATP1A2-mediated uptake of imatinib has been shown to be significantly inhibited by naringin, the constituent in citrus juice that has been implicated in OATP1A2 inhibition, suggesting that imatinib may be vulnerable to drug interactions that inhibit OATP1A2 (Bailey et al., 2007; Yamakawa et al., 2011). In addition, there have been a number of polymorphisms that have been identified in the *SLCO1A2* gene, which encodes OATP1A2 (Franke et al., 2009) and *SLCO1A2* SNPs that may affect the disposition of imatinib have recently been identified (Yamakawa et al., 2011). Considering that green tea extract is commonly used as a supplement and is currently under investigation for treatment in various cancers, the likelihood that green tea will be used with numerous concomitant medications is high. Also considering that currently available drugs and new molecular

entities are increasingly being recognized as substrates for transport, the potential for adverse drug interactions is present.

In conclusion, this study indicates that green tea extract significantly alters fexofenadine pharmacokinetics, most likely as a result of OATP1A2 inhibition in the intestine. These findings have clinical importance as green tea extract may alter the disposition of other transporter substrates. Further studies are warranted to evaluate the role of green tea extract in other potential drug interactions that may result in reduced efficacy or increased toxicity.

Table 4-1. Effect of green tea extract on fexofenadine pharmacokinetics parameters after a single 60mg oral dose of fexofenadine to 8 healthy volunteers

Parameters	Control phase	EGCG phase	Mean difference	95 % CI	P value
t_{max} (h)	2.88 ± 1.13	2.13 ± 0.83	-0.75 ± 0.54	(-1.68, 0.18)	NS
C_{max} (ug/L)	146 ± 56.9	50.2 ± 25.2	-95.5 ± 40.3	(-165, -25.8)	0.0154
λ_z (h^{-1})	0.0894 ± 0.01	0.0895 ± 0.02	0.0001 ± 0.015	(-0.026, 0.026)	NS
$t_{½}$(h)	7.82 ± 0.79	8.05 ± 1.60	0.23 ± 1.22	(-1.88, 2.34)	NS
V_{ss} (L)	452 ± 132	1527 ± 816	1076 ± 457	(285, 1866)	0.0158
AUC_{0-24} (ug h/L)	812 ± 289	269 ± 133	-544 ± 193	(-877, -210)	0.0072
$AUC_{0-\infty}$ (ug h/L)	872 ± 303	294 ± 137	-578 ± 196	(-916, -239)	0.0058
CL/F (L/h)	75.5 ± 22.7	249 ± 119	174 ± 64.1	(62.8, 285)	0.0086

Data expressed as mean ± standard deviation,

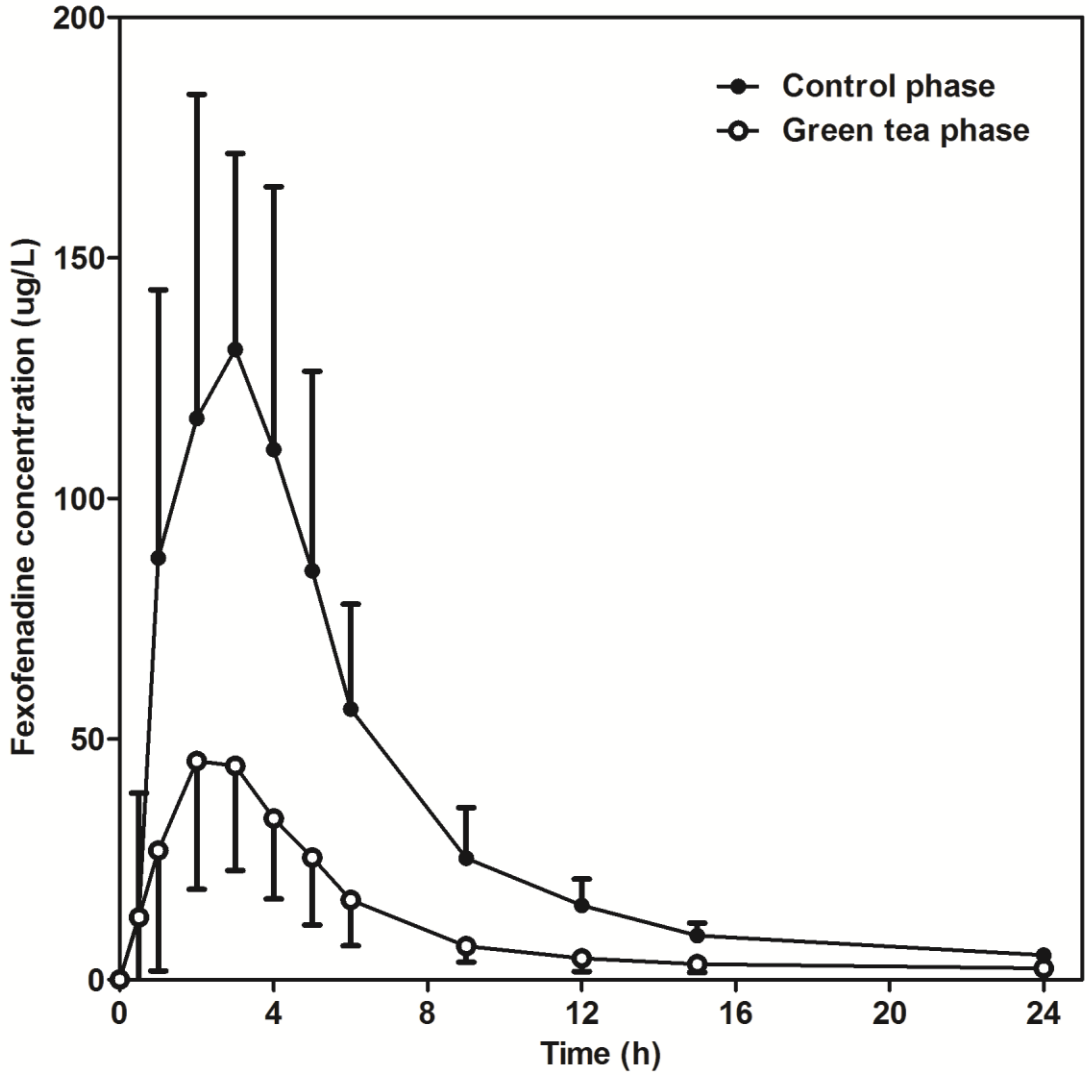

Figure 4-1. Mean (± standard deviation) concentration-time profiles for the control and green tea phases

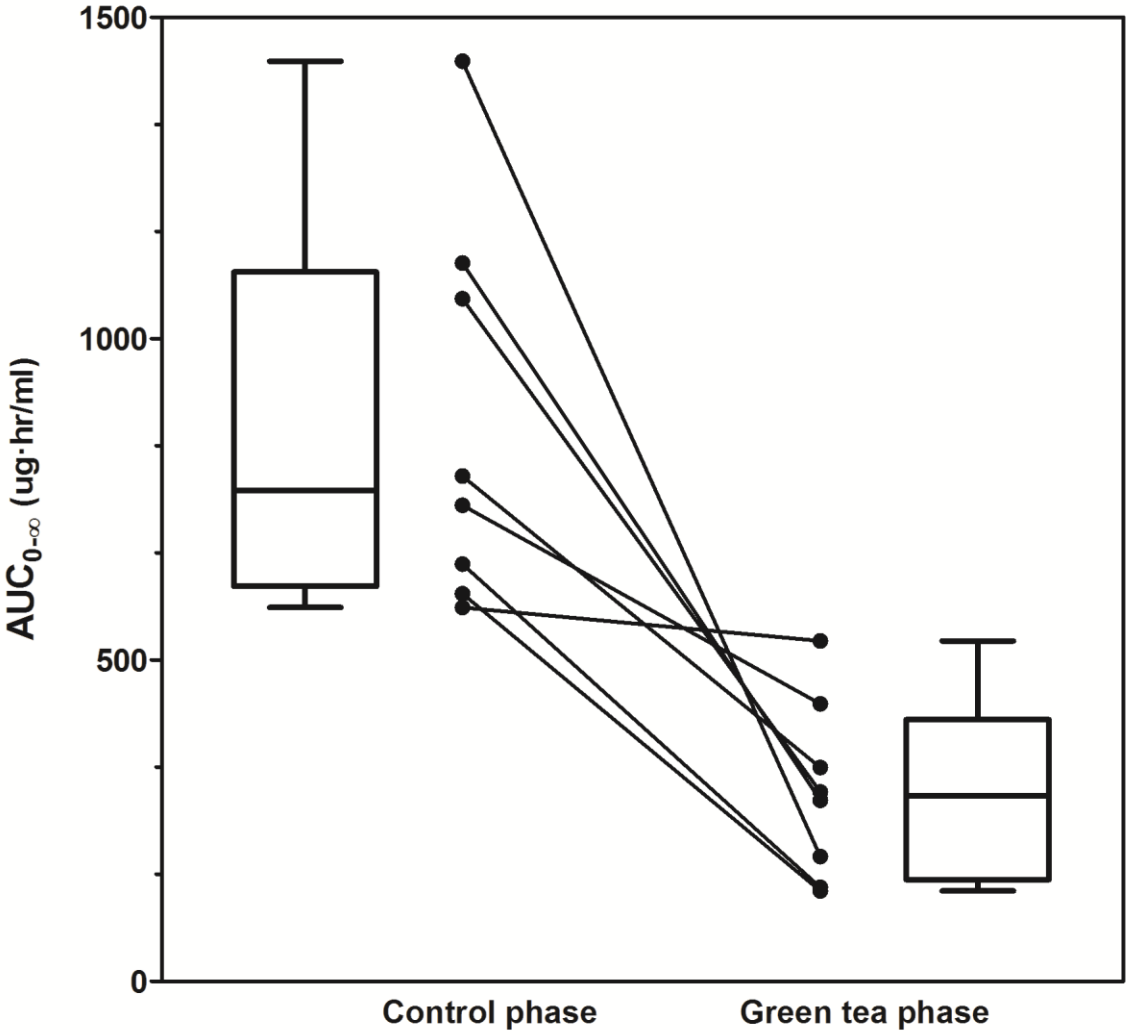

Figure 4-2. Individual, median, and interquartile range of fexofenadine AUC0-∞ values in the control and green tea phases

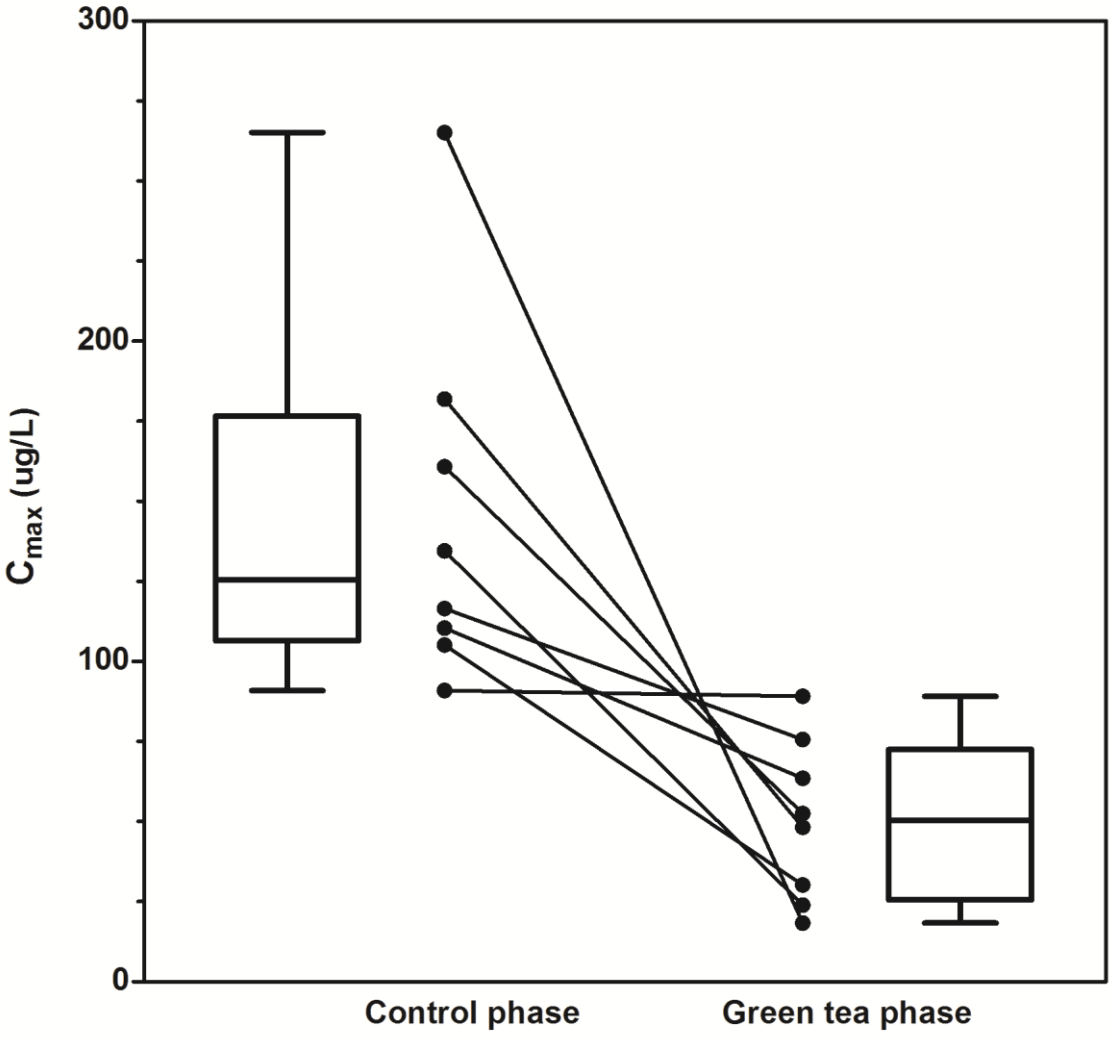

Figure 4-3. Individual, median, and interquartile range of fexofenadine C_{max} values in the control and green tea phases

CHAPTER 5
CONCLUSION AND FUTURE DIRECTIONS

The goal of this research was to evaluate the effects of genetic variability and a botanical interaction on drug disposition and response. This research has provided important new insight into the role of OATP-mediated transport. Further, the results of this work will generate new hypotheses and research questions to investigate the effects of disease, genetic variation, and other concomitant drugs/herbs/foods on the function of OATP-mediated transport. Fully understanding how these factors affect OATP-mediated transport will ultimately help guide us in predicting clinical efficacy, toxicity, and drug-drug interactions.

First, we investigated the effect of genetic variability on OATP-mediated uptake and drug response. Specifically, we evaluated the effect of *SLCO1B1*, the gene that encodes OATP1B1, reduced-function haplotypes on atorvastatin response. OATP1B1 facilitates the hepatic uptake of atorvastatin, allowing for access to the site of action as well as elimination, and polymorphisms within *SLCO1B1* have the potential to affect both the disposition and the response of statins and other substrate drugs. Therefore, we hypothesized that OATP1B1 reduced-function carrier status is associated with diminished lipoprotein reduction and enhanced inflammatory marker reduction. To test this hypothesis, we conducted a retrospective pharmacogenetic analysis in 81 normocholesterolemic individuals without cardiovascular disease who received atorvastatin 80 mg/day orally for 16 weeks. Genotypes of the 388A>G and 521T>C SNPs were determined for each subject and haplotypes were assigned and categorized as either a carrier or non-carrier of a reduced-function OATP1B1 haplotype. Percent changes of lipoprotein and inflammatory marker concentrations from baseline to week

16 were determined and compared between OATP1B1 reduced-function carrier and non-carrier groups. In this analysis, we did not find an association between OATP1B1 reduced-function carrier status and atorvastatin response. However, due to the design of the original clinical study, we did not have pharmacokinetic data and were not able to make any conclusions regarding the effect of the OATP1B1 reduced-function carrier status on atorvastatin disposition and how that may relate to atorvastatin response. Also, this study was conducted in healthy individuals and may not be representative of patients who are treated with statins. As OATP1B1 is an important determinant in the disposition of statins as well as many other substrate drugs, future research is still warranted to evaluate the effects of genetic variability on OATP-mediated uptake in other populations and other substrate drugs.

Next, we evaluated the effect of green tea extract (EGCG) on in vivo OATP-mediated uptake. We hypothesized that EGCG inhibits in vivo OATP-mediated uptake based on previous in vitro studies demonstrating the inhibitory effects of EGCG on OATP-mediated transport. This interaction, to our knowledge, has not been previously investigated in vivo. Thus, we evaluated the in vivo effect of EGCG on OATP-mediated transport for the first time in a clinical pharmacokinetic drug interaction study with the OATP probe fexofenadine in healthy volunteers. In order to measure fexofenadine concentrations, we developed and validated a specific and sensitive LC/MS/MS assay (Chapter 2). The clinical study was a randomized open-label crossover study consisting of a control phase and a green tea (EGCG) phase, in which the subjects received either a single dose of fexofenadine 60 mg alone or after a single dose of green tea extract (EGCG 800 mg). Blood samples were collected over 24 hours during each phase and

fexofenadine pharmacokinetic parameters were compared between the two phases. We found that fexofenadine plasma concentrations were significantly reduced in the green tea phase as compared to the control phase. Specifically, green tea extract reduced fexofenadine $AUC_{0-\infty}$ and C_{max} and increased CL/F and V_{ss} without change in $t_{1/2.}$ These results show that green tea extract reduces fexofenadine bioavailability suggesting inhibition of intestinal OATP-mediated uptake. As OATP-mediated transport is an important determinant of disposition and response for many drugs, these findings warrant further research to identify other potential drug interactions.

In conclusion, the effects of genetic variation and green tea extract on OATP-mediated transport have been studied. Although we were unable to detect differences in atorvastatin response between OATP haplotype groups, further research is still warranted as genetic variation in OATP-mediated transport has been shown to be important in statins as well as other substrate drugs. However, we did show that the botanical green tea extract alters the disposition of the OATP probe fexofenadine, suggesting inhibition of OATP-mediated uptake in the intestine. Future research is needed to further evaluate the role of green tea extract and other botanicals in drug interactions as it could have significant clinical implications for drug therapy.

APPENDIX
SUPPLEMENTAL DATA FROM FEXOFENADINE-GREEN TEA EXTRACT CLINICAL STUDY

Table A-1. Individual fexofenadine pharmacokinetic parameters

Subject	Control phase		Green tea phase	
	$AUC_{0-\infty}$ (ug·h/L)	C_{max} (ug/L)	$AUC_{0-\infty}$ (ug·h/L)	C_{max} (ug/L)
1	786.26	110.41	332.72	63.51
2	740.96	116.56	431.81	75.63
3	603.07	105.19	140.91	30.28
4	581.94	90.97	529.64	89.14
5	1431.80	265.11	194.31	18.40
6	1117.76	181.98	281.54	48.27
7	1062.34	160.84	294.81	52.57
8	649.27	134.57	146.33	23.92

Table A-2. Subject demographics

Subject	Age (yrs)	Height (cm)	Weight (kg)	Race (self-reported)
1	20	175.7	73.2	White
2	20	170.5	74.2	African American
3	21	162.7	65.2	White, Hispanic
4	58	170.3	75.9	White
5	54	164.3	69.5	African American
6	21	163.4	77.9	White, Hispanic
7	33	169.1	64.0	Asian
8	20	170.1	66.4	White, Hispanic

Table A-3. Individual fexofenadine pharmacokinetic parameters for subjects who received half-dose green tea extract

	Subject A		Subject B	
Parameter	Control phase	Green tea phase	Control phase	Green tea phase
t_{max} (h)	4.00	1.00	1.00	1.00
C_{max} (ug/L)	45.71	62.86	189.75	154.07
λ_z (h^{-1})	0.1016	0.0833	0.1140	0.1164
$t_{½}$(h)	6.82	8.32	6.08	5.95
V_{ss} (L)	1233.00	1090.16	313.99	401.08
AUC_{0-24} (ug h/L)	355.13	279.43	1102.02	737.21
$AUC_{0-\infty}$ (ug h/L)	391.34	303.69	1156.22	770.79
CL/F (L/h)	153.32	197.57	51.89	77.84

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BIOGRAPHICAL SKETCH

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